THE ROLE OF UNESTERIFIED FATTY ACID TRANSPORT IN CHYLOMICRON METABOLISM

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In a study to determine the early fate of absorbed fat, previously reported from this laboratory, observations were made on the removal of chylomicra containing triglyceride labeled with palmitic acid-1-C^{14} from the plasma of the anesthetized dog (1). It was found that the triglyceride fatty acids (TGFA) rapidly appeared in the albumin-bound unesterified fatty acid fraction (UFA) of the plasma. The turnover of plasma UFA also was estimated from the rate of disappearance of palmitate-C^{14} immediately after injection of the albumin-bound form and was found to be very high. It was concluded that the clearing of chylomicra was intimately associated with hydrolysis of the triglycerides and retransport of their constituent fatty acids in plasma as UFA. Since these experiments did not include measurement of oxidation of the administered fatty acids it was not possible to assess the relative importance of the UFA pathway in the subsequent metabolism of TGFA.

The experiments described in the present paper were made primarily to investigate this relationship. For this purpose, the oxidation and plasma turnover of C^{14}-palmitate administered as TGFA and as UFA have been compared in carefully controlled experiments.

METHODS

Substrates. Chylomicra containing palmitate-1-C^{14}-labeled fatty acids were obtained from donor dogs prepared as previously described (1). Chylomicra as defined here included that lipoprotein fraction which packed firmly at the top of the tube after chyle was layered under saline at density 1.006 and centrifuged for 30 minutes at 100,000 × G. The material obtained in this way and washed twice more in saline by the same process consisted of more than 90 per cent triglyceride and 0.5 to 1 per cent protein. Chemical analyses (*vide infra*) indicated that 98 to 99 per cent of the radioactivity was present in the triglyceride fraction and that no more than 0.2 per cent was present as unesterified fatty acid.

The total lipid in a single administration of chylomicra was 13 to 80 mg. per kilogram body weight (Table I), suspended in 20 ml. of saline and injected intravenously over a 10 to 15 second period. This amount of lipid provided the minimum amount of radioactivity necessary for extended measurements of blood UFA radioactivity, but probably exceeded the usual physiological rate of entry of chylomicra into the blood since the average donor animal used delivered about 5 mg. of chylomicron lipid per kilogram body weight per minute from the thoracic duct fistula. In one experiment 1.5 mg. of lipid per kilogram per minute was infused continuously for a two hour period.

Palmitic acid-1-C^{14} was obtained from the Nuclear Instrument and Chemical Company, Chicago. Counter current distribution indicated that approximately 95 per cent of the radioactivity was present in palmitic acid, the remainder in highly polar acids. Two preparations were used, having approximate specific activities of 2.54 and 20.0 mc. per mM.

Unesterified palmitic acid-1-C^{14} was prepared as the albumin complex as follows: Approximately 6 mg. (0.02 milliequivalent) of the potassium salt was dissolved in ethanol. Three ml. of isotonic saline was added and the ethanol evaporated. The warm saline solution was mixed quickly with 20 ml. of dog plasma, sufficient to provide an estimated equimolar ratio of albumin to UFA. The serum utilized was obtained from a dog 30 minutes after the administration of glucose and insulin to obtain the lowest possible concentration of endogenous UFA. The total quantity of unesterified fatty acid injected in the optically clear solutions represented roughly 2 to 4 per cent of the amount present in the total plasma volume of the recipients.

Recipients. The recipients were either male or female mongrel dogs. "Carbohydrate loaded" dogs (Table I) were given a diet consisting of dextrose in water and low-fat bread for four days and an infusion containing 25 grams of glucose and 10 units of regular insulin for 30 minutes preceding the experiment. "Fasted" animals were fed a diet consisting of bacon for four days followed by the withdrawal of all food for 24 hours. A saline infusion was substituted for the dextrose given the "carbohydrate-loaded" animals, and all animals received isotonic saline at a rate of 0.5 ml. per minute following injection of the labeled substrates. The dogs were maintained in light anesthesia throughout the experiment by intravenous administration of sodium Pentothal®.

An endotracheal tube connected to a low resistance

two-way valve was utilized for collection of expired air in Douglas bags. Immediately preceding the injection of the labeled substrate, a sample of air was collected for determination of the respiratory quotient. Blood samples were collected rapidly through an in-lying needle in the femoral artery. Either dried balanced oxalate or heparin was used as anticoagulant. The bloods were immediately chilled and kept at ice temperature.

Separation of chylomicra and UFA. In the experiments in which chylomicron disappearance was measured, the chylomicra were separated by ultracentrifugation of blood, rather than plasma, in order to reduce the number of manipulations involved. Recovery of chylomicra was found to be comparable to that obtained when equivalent amounts of plasma were used. Five ml. aliquots of whole blood were layered under isotonic saline and centrifuged for 18 hours at 105,000 × G in a Spinco No. 40 rotor at 5° (2). The tubes were then sliced in the clear and colorless zone just below the visible fatty layer at the top. The chylomicra and lipoproteins of density less than 1.006 contained in the supernate were carefully resuspended in saline in graduated vessels. The infranates, including the packed red cells, were resuspended and lyophilyzed without delay for subsequent extraction of UFA. During the course of these experiments it was found that after injection of labeled chylomicra or UFA, about 2 per cent of the total radioactivity and about 15 per cent of the total titratable acidity found in the UFA extracts could not be removed from the suspended cells by three washes with isotonic saline. Since this might have tended to lower blood UFA specific activity below that of plasma, several sets of experiments comparing the metabolism of labeled TGFA and UFA were also performed in which plasma was ultracentrifuged and the UFA extracted from the infranates.

Chemical analyses. Aliquots of the substrate chylomicron suspensions and UFA-albumin solutions were extracted in acetone : ethanol (1 : 1). The supernatant lipoproteins from the ultracentrifugal separations were extracted in chloroform : methanol (2 : 1). Aliquots were removed for appropriate lipid analyses (3–5) and for assay of radioactivity. Unesterified fatty acids were extracted from the infranates obtained from the ultracentrifugal runs, whole blood or plasma, and red cell suspensions according to the method of Gordon (6). The unesterified fatty acids were further separated from the neutral lipid in the iso-octane phase by extraction with 1 M NaOH in 50 per cent ethanol, followed by acidification and re-extraction of the acids into iso-octane according to the method of Borgström (7). The final phase was washed three times with a 1 : 2,000 solution of H_2SO_4 before aliquots were removed for titration and counting.

Borgström has reported that his extraction procedure could result in contamination of the UFA phase with lower glycerides. Laurell (8) has also raised the possibility that extraction with unbuffered alkali might produce free fatty acids through hydrolysis of neutral lipid, especially phospholipid. While the original iso-octane extract contained less than 5 per cent of the plasma phospholipids, several other procedures were utilized to ascertain whether the small amounts of labeled UFA observed in the late course of these experiments could be spuriously produced by the method of extraction employed, or represent radioactivity in lower glycerides contained in the "UFA extract."

Test mixtures containing purified palmitic acid, tri-, di-, and monopalmitin were taken through the combined extraction procedures. The final "UFA extract" contained 99 per cent of the added palmitic acid, 10 per cent of added monopalmitin, and less than 1 per cent of the added higher glycerides. An additional purification step was therefore utilized in two experiments (Dogs C-5 and U-9) to exclude the possibility of contamination of the UFA fraction by lower glycerides. In these experiments, the 15 ml. "UFA extracts" were shaken with 5 grams of silicic acid in 50 ml. extraction tubes. After centrifugation, the iso-octane was decanted. Exactly 20 ml. of 15 per cent diethyl ether in iso-octane was shaken with the silicic acid, the suspension again centrifuged, and the solvent decanted. It was determined separately that such an eluate contained approximately 50 per cent of cholesterol palmitate, tripalmitin, labeled palmitic, oleic and linoleic acids previously adsorbed on the silicic acid. No monopalmitin, crude lecithin or cephalin added to the silicic acid could be detected in the eluates. The UFA specific activities obtained with these purified extracts did not differ significantly from those obtained using the simpler combined extraction procedures.

Finally, plasma was obtained from a dog 5 and 120 minutes after the injection of 50 μc. of palmitic acid-1-C^{14}. The plasma was extracted in acetone : ethanol (1 : 1), the solvent evaporated *in vacuo*, and the lipids taken up in ether previously equilibrated with water. The ether solution was then passed through a column containing Amberlite IRC-400, after the method of Savary and Desnuelle (9). All neutral lipids, including monopalmitin and lecithin, were quantitatively eluted from the resin by ether, while 99 per cent of added palmitic acid-1-C^{14} was retained. The amounts of UFA radioactivity determined by this procedure in the 5 and 120 minute plasma samples did not differ significantly from those obtained in the other experiments where the combined solvent extractions were used.

The concentrations of UFA obtained by titration were corrected to milliequivalents per liter of whole blood or plasma. Estimation of specific activity of the chylomicron triglyceride fatty acids was made by assumption of the mean molecular weight of 260 for the fatty acid moiety. The distribution of radioactivity in the chylomicron lipid was determined by silicic acid chromatography by the method of Hirsch and Ahrens (10).

Radioactivity measurements. Carbon-14 measurements were made at − 10° C. in a Packard Tri-Carb Scintillation Spectrometer. Aliquots of lipid extracts were dried in vials and 15 ml. of phosphor solution (400 mg. per cent diphenyloxazole in toluene) added. In the assay of samples containing only UFA, 50 mg. of carrier palmitic acid was added to the aliquots before evaporation

at steam bath temperature. This amount of material was found to minimize loss of UFA by sublimation without the introduction of quenching.

Total and specific radioactivity of carbon dioxide-C^{14} in expired air samples collected for 1 to 5 minute periods was determined after direct collection in methyl benzethonium (11). The samples were corrected for quenching to permit comparison with the radioactivity in the lipid samples.

All values for chylomicron, UFA and carbon dioxide radioactivity were further adjusted, by a factor derived from the total radioactivity administered and the weight of the recipient, to a value corresponding to a dose of 10^6 cpm per kilogram body weight.

RESULTS

1. Chylomicron clearance

The disappearance of radioactivity from the fraction of blood containing chylomicra and other lipoproteins of density less than 1.006 is shown in Figure 1, for both fasted and carbohydrate-fed recipients. The disappearance patterns were reasonably similar. Each curve was complex, and included an initial rapid phase which approximated a straight line when the logarithm of total blood chylomicron radioactivity was plotted against time. During this phase the radioactivity fell to about 10 per cent of the zero time value, with 2.5 to 4.5 minutes required to reach half maximal concentration. Following this, the radioactivity disappeared more slowly for 30 to 60 minutes, beyond which accurate measurements could not be made.

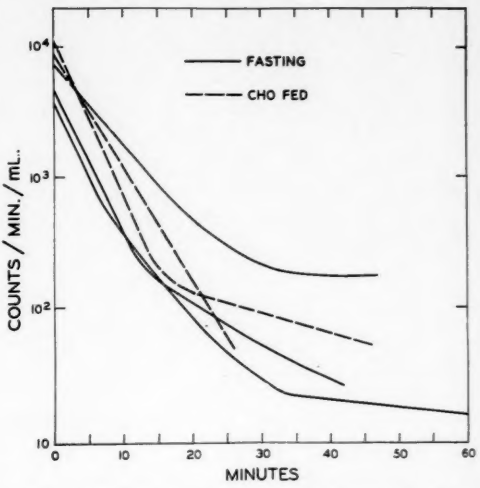

FIG. 1. THE DISAPPEARANCE OF PALMITATE-1-C^{14}-LABELED CHYLOMICRA FROM THE BLOOD AFTER FASTING (DOGS C-1,-2 AND -3) AND AFTER CARBOHYDRATE FEEDING (DOGS C-1 AND -2)

The lower four curves were obtained from Dogs C-1 and C-2 (Table I).

2. Appearance of radioactivity in the blood UFA

Radioactivity appeared very quickly in the blood UFA after the injection of labeled chylomicra (Figure 2). The time, t_{max}, when peak specific activity was reached, occurred within 6 to 10 minutes, or after about three-quarters of the injected triglyceride had been removed from the blood. In two dogs (C-1 and C-2) the appearance of labeled UFA was compared after both fasting and carbohydrate feeding (Figure 2). Carbohydrate feeding did not delay the occurrence of t_{max}, but the maximum concentration of labeled UFA present was only half of that obtained in the fasting state. From the rapid rate with which injected labeled UFA was removed from the blood (Figure 4) it could be ascertained that the small amount of "preformed" UFA-C^{14} injected with the chylomicra did not contribute significantly to the levels of radioactivity in UFA arising from TGFA.

3 The contribution of chylomicron TGFA to circulating blood UFA

The peak specific activity of the UFA in blood after injection of labeled palmitate as chylomicron TGFA varied from 6 to 15 per cent of the calculated specific activity of the infused triglyceride fatty acids. The ratio of peak UFA specific activity to substrate specific activity was much less than had been previously obtained with injection of larger amounts of chylomicra (1).

An experiment was performed to estimate the contribution which fatty acids arising from continuously infused chylomicron triglyceride might make to blood UFA. A fasting dog was given approximately 1.5 mg. of labeled chylomicron TGFA per kilogram body weight per minute by intravenous infusion lasting 120 minutes. After one hour, the blood chylomicron radioactivity and the concentration and specific activity of the blood UFA stayed at constant levels. During this time the UFA specific activity was approximately 10 per cent of that of the infused TGFA-C^{14}. In this

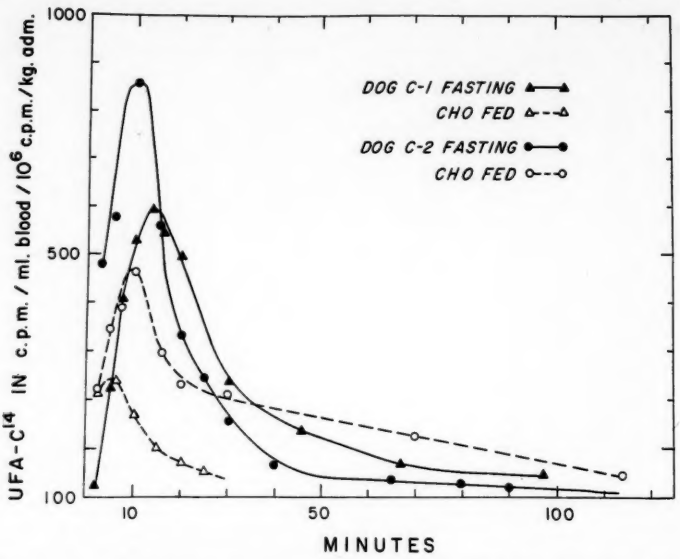

FIG. 2. THE APPEARANCE OF RADIOACTIVITY IN BLOOD UFA AFTER ADMINISTRATION OF PALMITATE-1-C^{14} AS CHYLOMICRON TGFA

simulation of physiological fat absorption, then, not more than 1 UFA molecule out of 10 in the blood appeared to be coming from the infused chylomicron TGFA.

4. The oxidation of chylomicron TGFA

As was reported in a preliminary communication (12), the oxidation of palmitic acid-1-C^{14} injected as chylomicron TGFA began without significant delay. Similar findings have also been reported in the rat (13, 14). Specific activity-time curves for expired $C^{14}O_2$ are shown in Figure 3. Maximum specific activity was reached 35 to 70 minutes after injection, and was followed by a slow steady decline. In the same two dogs compared after both fasting and carbohydrate feeding, the maximum outputs of labeled carboxyl carbon (in counts per minute per minute) were increased 3.6-fold (Dog C-1) and 1.7-fold (Dog C-2) by fasting. Thus, as the data in Tables I and II also indicate, a dog such as C-1, tested under extreme differences in nutritional state, tended to spare triglyceride fatty acids when adequate glucose was available.

To determine the relationship of labeled carboxyl carbon appearing in the blood as UFA to that appearing in the expired CO_2 the metabolic behavior of labeled palmitate injected as UFA was next examined.

5. Metabolism of albumin-bound palmitic acid-C^{14}

The disappearance of labeled palmitic acid from the blood after intravenous injection as UFA is shown in Figures 4 and 5. The form of the disappearance curves in dogs was very similar to that observed in man. An interpretation of UFA metabolism, based on similar tracer data, has been presented elsewhere (12, 15). In these experiments, when the logarithm of UFA radioactivity is plotted against time, the earliest portion of the curve approximates a straight line for 2 to 5 minutes. A fractional turnover rate, k, for disappearance of UFA from the blood may be derived from the slope of this line. From the product of k(minutes^{-1}) × UFA concentration (mEq. per liter) × plasma volume (liters) a plasma turnover of UFA (in mEq. per minute) is obtained. This estimation includes the assumption that all UFA in the blood disappears at the rate observed for the tracer acid. A similar turnover rate, which is dependent on the UFA concentration, has been observed for labeled palmitic, oleic and linoleic acids in man

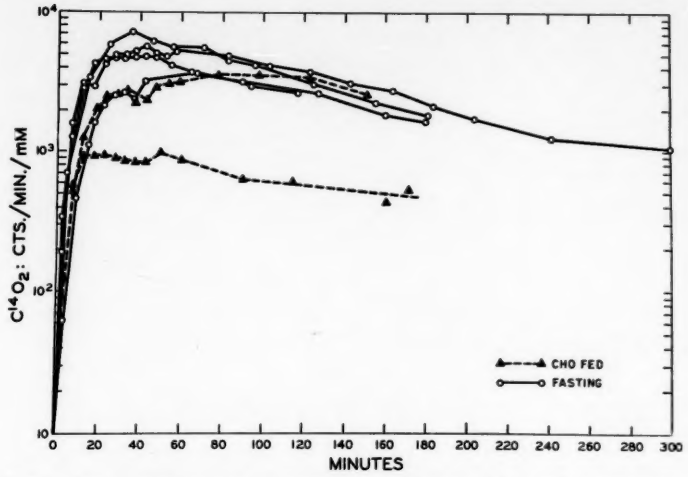

FIG. 3. THE APPEARANCE OF RADIOACTIVITY IN EXPIRED CO_2 AFTER ADMINISTRATION OF PALMITATE-1-C^{14} AS CHYLOMICRON TGFA

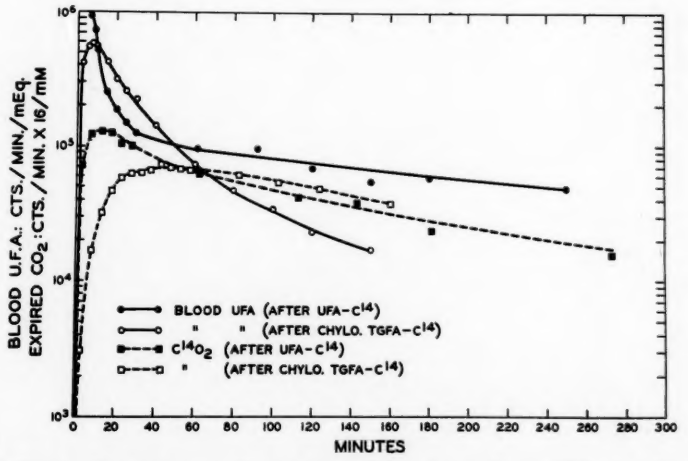

FIG. 4. COMPARISON OF RADIOACTIVITY OBTAINED IN BLOOD UFA AND EXPIRED CO_2 AFTER ADMINISTRATION OF PALMITIC ACID-1-C^{14} AS CHYLOMICRON TGFA AND AS UFA

(15). The later complexity of the UFA disappearance curve has been interpreted to result from recycling of labeled UFA molecules back into the plasma (15).

The specific activity of labeled CO_2 in the expired air after the albumin-palmitate complex was injected is also shown in Figure 4. From Table II it can be seen that 20 to 30 per cent of the UFA carboxyl carbon was excreted as CO_2 within three hours after first entering the plasma of fasting dogs.

6. Differences in the metabolism of palmitic acid injected as TGFA or UFA

Since it may be assumed that, once a labeled palmitate molecule is present in the plasma as UFA, its subsequent metabolic fate will be independent of its origin, it should be possible to as-

TABLE I

Status of animals receiving palmitate-1-C^{14} as chylomicron triglyceride or as albumin-bound unesterified fatty acid (UFA)

Dog	Wgt.	Form of palmitate-1-C^{14}	Nutritional status	Respiratory quotient	Plasma UFA	Total lipid infused
	Kg.				*mEq./L.*	*mg./Kg.*
C-1	15.6	Chylomicron TGFA*	Fasted	0.6	1.78	70
	15.3		Carbohydrate fed	1.0	0.41	63
C-2	16.7	Chylomicron TGFA	Fasted	0.6	1.46	65
	16.7	Chylomicron TGFA	Carbohydrate fed	0.9	0.97	68
C-3	20.0	Chylomicron TGFA	Fasted	0.6	0.64	45
C-4	14.3	Chylomicron TGFA	Fasted	0.6		13
C-5	11.5	Chylomicron TGFA	Fasted	0.8	0.70	67
U-7	12.3	UFA	Fasted	0.6	0.77	<1
	14.9	UFA	Carbohydrate fed	1.0	0.41	<1
U-8	16.5	UFA	Fasted	0.6	0.50	<1
U-9	16.5	UFA	Fasted	0.8	0.67	<1 (UFA) 65 (TGFA)

* Triglyceride fatty acid.

certain from these data whether or not retransport as UFA must occur before chylomicron triglyceride fatty acids are oxidized. This may be approached in two ways: *1*) by a comparison of specific activities observed in blood or plasma UFA and expired CO_2, and *2*) by comparison of the turnover or flux of labeled UFA in the plasma with that observed in the expired CO_2 after injection of palmitate-C^{14} in the two substrate forms.

In Figure 4 are presented specific activities in UFA and CO_2 from one fasting dog (U-*7*) given UFA-C^{14} and composite data from three fasting dogs (C-1,-2 and -3) given labeled chylomicra. The $C^{14}O_2$ specific activities have been multiplied by a factor of 16 to approximate the minimal "obligatory dilution" assumed to occur during the oxidation of a singly-labeled palmitic acid molecule (16). From this figure it may be concluded that palmitic acid injected as either UFA or as chylomicron TGFA was oxidized at similar rates and apparently with similar dilution. As will be seen from the cumulative excretion of labeled carboxyl carbon (Table II) the early delay in reaching maximum specific activity in oxidation of TGFA represented the only difference. A second reasonable conclusion is that all chylomicron TGFA was not retransported in the blood as UFA before it was oxidized. This is based on the observation that the specific activity of the blood UFA derived from TGFA eventually dropped below that of the $C^{14}O_2$ and stayed there for several hours. During this time, the observed $C^{14}O_2$ specific activities could not have reasonably come solely from the oxidation of blood UFA in which the radioactivity was more dilute.

The specific activity data obtained in several sets of experiments were also used to calculate the

TABLE II

Total carboxyl-carbon-14 excreted by dogs in expired air as CO_2

Dog	Nutritional status	Form of palmitate-1-C^{14}	Percentage of radioactivity excreted as $C^{14}O_2$	
			30 min.	180 min.
			%	%
C-1	Fasting	TGFA	1.6	19
C-2	Fasting	TGFA	2.7	16
C-3	Fasting	TGFA	2.2	20
C-4	Fasting	TGFA	3.9	28
C-5	Fasting	TGFA	1.1	22
U-7	Fasting	UFA	9.8	23
U-9	Fasting	UFA	8.8	29
C-1	Carbohydrate fed	TGFA	0.9	5
C-2	Carbohydrate fed	TGFA	0.7	15
U-7	Carbohydrate fed	UFA	5.4	13

flux of labeled carboxyl carbon from plasma as UFA and in expired air as CO_2. Data calculated in this way are shown in Figures 5 and 6 for Dogs U-9 and C-5. Dog U-9 also received unlabeled chylomicra simultaneously with UFA-C^{14} (Table I) to permit identical comparison with C-5. The fluxes from plasma as UFA (in counts per minute per minute) were obtained by multiplying the UFA specific activities at time, t (in counts per minute per mEq.), by the plasma UFA turnover (in mEq. per minute) estimated from the plasma UFA "pool" and the turnover rate obtained from the initial slope of the disappearance curve for palmitate-C^{14} injected as UFA. Since the plasma UFA concentration in the dog receiving labeled TGFA (Figure 6) was the same, it was possible to use the same fractional turnover rate for the flux calculation in this animal (15). The flux of radioactivity in the CO_2 was calculated by multiplying the specific activities (in counts per minute per mM) by the mean rate of CO_2 output (in mM per minute). A curve was fitted to each plot of flux against time, and an analytical expression consisting of a sum of exponential terms derived. These expressions were then integrated to obtain the total flux during a six hour period of observation. The results of such calculations for the dogs shown in Figures 5 and 6 and for another pair of animals are shown in Table III. It is apparent that the amount of labeled UFA which cycled through the plasma after injection of TGFA-C^{14} was considerably less than after injection of UFA-C^{14} even though comparable amounts of carboxyl carbon were being excreted. Were all of the chylomicron triglyceride fatty acids to have been retransported as UFA before oxidation, the flux of radioactivity in the plasma UFA should have been essentially the same after palmitate-1-C^{14} was injected in either form. These data are therefore incompatible with the obligatory retransport of chylomicron triglyceride fatty acids

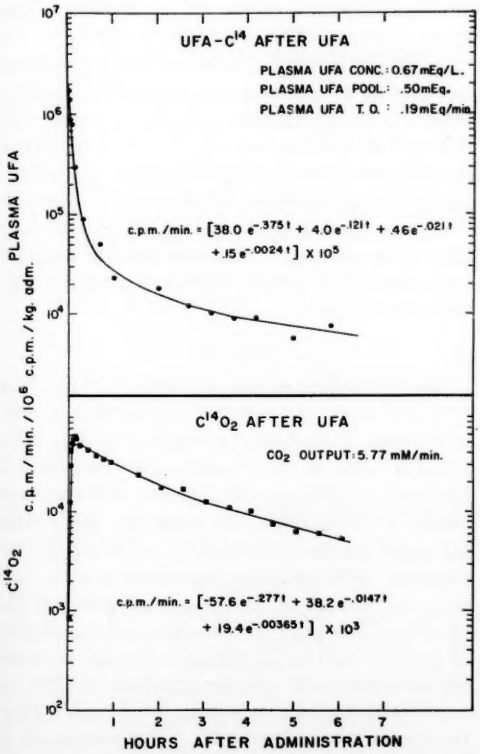
UFA-C14 AFTER UFA
PLASMA UFA CONC.: 0.67 mEq/L.
PLASMA UFA POOL: .50 mEq.
PLASMA UFA T.O. : .19 mEq/min.
c.p.m./min. = [38.0 e^-.375t + 4.0 e^-.121t + .46 e^-.021t + .15 e^-.0024t] x 10^5
C14O2 AFTER UFA
CO2 OUTPUT: 5.77 mM/min.
c.p.m./min. = [-57.6 e^-.277t + 38.2 e^-.0147t + 19.4 e^-.00365t] x 10^3
PLASMA UFA
C14O2
c.p.m./min. / 10^6 c.p.m./kg. adm.
HOURS AFTER ADMINISTRATION

FIG. 5. THE FLUX OF LABELED CARBON FROM PLASMA AS UFA AND IN EXPIRED AIR AS CO_2 AFTER ADMINISTRATION OF PALMITIC ACID-1-C^{14} AS UFA

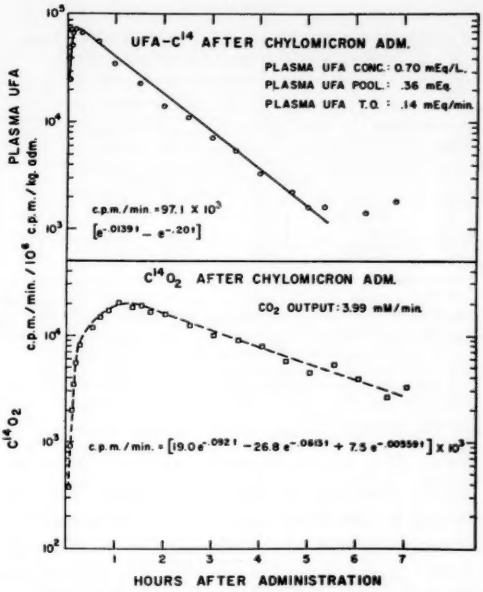
UFA-C14 AFTER CHYLOMICRON ADM.
PLASMA UFA CONC.: 0.70 mEq/L.
PLASMA UFA POOL: .36 mEq.
PLASMA UFA T.O. : .14 mEq/min.
c.p.m./min. = 97.1 x 10^3 [e^-.0139t - e^-.201t]
C14O2 AFTER CHYLOMICRON ADM.
CO2 OUTPUT: 3.99 mM/min.
c.p.m./min. = [19.0 e^-.092t - 26.8 e^-.0613t + 7.5 e^-.00559t] x 10^3
PLASMA UFA
C14O2
c.p.m./min. / 10^6 c.p.m./kg. adm.
HOURS AFTER ADMINISTRATION

FIG. 6. THE FLUX OF LABELED CARBON FROM PLASMA AS UFA AND IN EXPIRED AIR AS CO_2 AFTER ADMINISTRATION OF PALMITATE-1-C^{14} AS CHYLOMICRON TGFA

TABLE III

Comparison of the flux of labeled carboxyl carbon from palmitic acid in plasma UFA and expired CO_2

Dog	Substrate	Total dose C^{14}	Total C^{14} flux* in: Plasma UFA	Total C^{14} flux* in: Expired CO_2	Ratio flux UFA/CO_2
		cpm $\times 10^6$	*cpm* $\times 10^6$		
U-9†	UFA	16.5	19.3	6.3	3.1
C-5†	TGFA	11.5	6.5	3.7	1.8
U-1‡	UFA	12.3	12.6	3.0	4.0
C-4‡	TGFA	20.0	7.1	4.6	1.5

* Flux equals $\int_0^t \gamma \, dt$, where γ is the sum of exponential terms in the analytical expression derived from the plot of log cpm per minute against time; in plasma UFA-C^{14} ($\gamma = \gamma_a$); in expired $C^{14}O_2$ ($\gamma = \gamma_b$).
† Time period of observations, 360 minutes.
‡ Time period of observations, 240 minutes.

as UFA before oxidation. From the data in Table III it may be inferred that about half of the TGFA oxidized in these experiments had appeared in the blood as UFA. A more rigorous analysis of these and similar data to calculate the extent to which the UFA pathway is utilized and to estimate the rate of hydrolysis of the triglycerides is in preparation (17).

DISCUSSION

The present experiments were designed to determine the importance of the blood UFA transport system in the metabolism of chylomicron triglyceride. A large fraction of the chylomicron triglyceride fatty acids appeared to be oxidized without first reappearing in the plasma as UFA. In fasting dogs, however, palmitate-C^{14} injected as chylomicron TGFA or as UFA was oxidized at similar rates and yielded comparable specific activities in the expired CO_2, except for an initial lag following TGFA administration. This suggests that much of the triglyceride may have been directly removed and oxidized in the same tissue "pools" as the UFA. Recent experiments by Morris and French, using liver perfusion (18), indicate that this organ, at least, is capable of both removing and oxidizing chylomicron TGFA. Bragdon and Gordon have measured the tissue distribution of palmitate-C^{14} after intravenous injection as either TGFA or UFA in fasting rats (19). At 10 minutes the radioactivity from both substrates was found principally in liver and skeletal muscle. While it is possible that chylomicron TGFA may be removed at one site and transported elsewhere as neutral lipid, it was previously shown (1) that only small amounts of radioactivity appeared in other plasma lipoproteins after chylomicra were administered. It appears reasonable to conclude that chylomicra represent a plasma vehicle for transport of fat which is directly available for oxidation. These findings also emphasize the importance of examining the possible caloric contribution from fatty acids carried as triglycerides in other lipoproteins.

The effect of carbohydrate on chylomicron metabolism is only partially known. In two dogs studied here after both fasting and carbohydrate loading, the removal of chylomicra was not impaired. The rate of reappearance of triglyceride fatty acids in the blood as UFA, as measured by the time required to reach maximum radioactivity, was also not decreased by carbohydrate feeding, but the total amount of TGFA returned to the blood appeared to be considerably less. This apparent decrease in UFA production may be due to a widely distributed effect of carbohydrate or to shunting of much of the chylomicra to areas where UFA production may be especially diminished by carbohydrate excess. It is of interest in this regard that chylomicra appear to accumulate in the adipose tissue in the rat only after carbohydrate feeding (19), and that release of fatty acids from adipose, which is presumably preceded by hydrolysis, is greatly decreased after carbohydrate feeding (6).

SUMMARY

The metabolism of palmitic acid-1-C^{14} has been compared after intravenous administration to dogs in the form of chylomicron triglyceride fatty acids (TGFA) and as the albumin-bound unesterified fatty acid (UFA). The specific activities and fluxes of radioactivity observed in the plasma UFA and in the expired CO_2 following the injection of both substrates have been used to estimate the amount of TGFA retransported in the blood as UFA prior to oxidation. The oxidation of palmitic acid proceeded at a similar rate and yielded comparable specific activities in CO_2 regardless of the form injected. However, a large fraction of the oxidized TGFA did not appear in the plasma as UFA, indicating that retransport as UFA is not obligatory in the utilization of exogenous triglyceride. Carbohydrate feeding de-

creased both the oxidation and amount of TGFA returned to the blood as UFA.

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IN VITRO HYDROXYLATION OF STEROIDS BY WHOLE ADRENAL HOMOGENATES OF BEEF, NORMAL MAN, AND PATIENTS WITH THE ADRENOGENITAL SYNDROME [1]

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The partial or complete failure of the adrenal cortex to realize the biosynthesis of Δ^4-pregnene-11β, 17α, 21-triol-3, 20-dione (compound F) represents the basic defect in patients with the adrenogenital syndrome due to congential adrenocortical hyperplasia (1–5). The most common form of this disorder (with normal blood pressure and without undue salt loss) has been indirectly attributed to a deficiency of adrenal "21-hydroxylase" as witnessed by the large quantities of urinary 3α, 17α, 20α-pregnanetriol (hereafter designated "pregnanetriol") and the relatively small amounts of steroids with an oxygen function at carbon 21 (4, 6, 7).

The availability of small amounts of human adrenal tissue has made it possible to examine the hydroxylation of some steroid substrates by normal and diseased glands *in vitro*. Due to the difficulties of obtaining any but limited fresh human material, fresh beef and postmortem human adrenals have also been employed for purposes of comparison. The experiments to be described were designed to measure the introduction of hydroxyl groups into steroid nucleus at positions 11 and 21 only. The appropriate adrenal enzymatic systems will be termed "11-hydroxylase" and "21-hydroxylase" after Dorfman (8), although it may be that each comprises more than a single component (9).

MATERIALS AND METHODS

Two normal "fresh" human adrenal glands were obtained from children during renal surgery. Several beef adrenals were collected from a slaughter house within an hour after death. These were frozen immediately after removal and stored at $-10°$ C. until used. A single normal gland from a child was taken, within 3 hours after death due to complications during cardiac surgery. In addition, "normal" adult glands were collected at the autopsy table within 24 hours after death. All glands were kept frozen and employed within two weeks of collection.

Adrenal glands from two children with the adrenogenital syndrome were available. One gland (Bz) was removed from a two and one-half year old female pseudohermaphrodite who had never been treated with steroids; the excretion of 17-ketosteroids was 13.1 to 15.0 mg. per day and the pregnanetriol, 15.0 to 18.0 mg. per day (normal in this laboratory: less than 1.0 mg. of either at this age). The left gland (weight, 14.0 Gm.) was excised during an exploratory operation and frozen immediately. The diseased glands (Sh), kindly sent us by Doctor Thomas Sheppard of Washington University, Seattle, were removed 18 hours after death from an infant, aged 18 months, and stored in the frozen state. The latter patient was on small, inadequate doses of cortisone at the time of death; the urinary 17-ketosteroids were 2.2 mg. per day and the pregnanetriol was not measured. This infant had the "salt-losing" form of the disease; in our experience, this variety has always been characterized by greatly elevated quantities of urinary pregnanetriol (2).

The preparation of the tissue, conditions of incubation, addition of adenosine triphosphate (ATP), diphosphopyridine nucleotide (DPN) and fumarate, dialysis and extraction were according to Hayano and Dorfman (10, 11), except that the whole gland was used and methylene chloride was substituted for chloroform. These workers reported satisfactory activity with frozen glands upon the addition of ATP and DPN. A tissue to steroid ratio of approximately 1,000 to 1 was generally employed, although this was occasionally exceeded with postmortem material as detailed in the data. In several instances, adrenal homogenates were examined without additional substrate or incubation in order to measure the baseline quantities of some steroids.

Two steroids were added to the incubates, separately, as substrates: Δ^4-pregnene-17α-ol-3,20-dione (17 hydroxyprogesterone, designated "17-HP"), and Δ^4-pregnene-17α, 21-diol-3,20-dione (hereafter designated "11-desoxycortisol"). The total recovery, after the addition of 17-HP, was measured in aliquots of the extracts in methanol,

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by the ultraviolet absorption at 225, 240 and 255 mμ employing a correction factor according to Allen (12). When 11-desoxycortisol was the substrate, suitable aliquots of the entire extract were examined by the Porter-Silber reagent (13) in order to determine the total recovery. This amount represents the basis for the calculation of the percentage conversion.

The extracts of the incubates were applied to paper chromatograms. The products sought, depending upon the substrate employed, were cortisol, 11-desoxycortisol and Δ^4-pregnene-11β,17α-diol-3,20-dione (21-desoxycortisol); and for baseline studies, in addition to the preceding compounds, 17α-hydroxyprogesterone. Several paper chromatograms were employed for each extract so that the quantity of steroid applied did not exceed 0.5 mg. per sheet, 6 inches in width. Suitable standards were always included along a narrow edge (0.5 inch) and the descending technique was employed as described elsewhere (14). Two solvent systems were used: for the measurement of cortisol and 11-desoxycortisol, system E6, containing 335 ml. iso-octane, 165 ml. toluene, 175 ml. tert.-butanol, 175 ml. methanol, and 100 ml. water; and for 17-hydroxyprogesterone and 21-desoxycortisol, system E4 (14), containing 500 ml. benzene, 250 ml. methanol, and 250 ml. water. All solvents were redistilled.

The standard compounds and products of incubation resolved by chromatography were located by scanning with ultraviolet light, the Zimmermann reaction as modified in this laboratory (15), triphenyltetrazolium hydrochloride and 4 per cent phosphomolybdic acid in methanol. Further details of this aspect appear elsewhere (16). The portions of the paper containing the extracts, where steroid was detected along a carefully measured narrow strip, were eluted three times with methanol. The quantities of cortisol and 11-desoxycortisol were measured by the Porter-Silber reaction (17) on small aliquots; the 21-methyl steroids were quantitated by the Gornall reaction (18) at the wave length of maximal absorption. Application of pure standards of each compound to paper chromatograms, resolution and elution, as described, yielded recoveries of 68 to 95 per cent.

TABLE I

Steroid content of adrenal glands in micrograms per gram tissue

Source	Condition	F*	S†	17-HP‡
Beef	N§ fresh frozen	8.9	11.6	0
Human	N fresh frozen (child)	4.1	5.6	0
Human	N postmortem (adult)	6.0	5.1	4.5
Bz	ADH‖ fresh frozen	0	0	118.5¶
Sh	ADH postmortem	0	0	155.5¶

* F, 17-hydroxycorticosterone.
† S, 17-hydroxy, 11-desoxycorticosterone.
‡ 17-HP, 17-hydroxyprogesterone.
§ N, normal.
‖ ADH, congenital adrenal hyperplasia.
¶ Identified (see text).

The identity of the products depended upon various criteria. The mobility on the chromatograms, the reactions with the reagents applied, and the sulfuric acid chromogens conformed in all instances to the designated compounds. In order to obtain the sulfuric acid chromogens, the eluates from paper were further purified on a small column of silica as described previously (16). In a few specified instances, enough steroid was obtained singly or in pairs of experiments for identification by infrared spectroscopy.

Homogenates of freshly frozen beef adrenal were included in all groups of experiments as an internal control. Several preliminary experiments with beef adrenal (not reported here) were conducted in order to standardize the conditions for this study.

RESULTS

Steroids in adrenal gland

The quantity of cortisol, 11-desoxycortisol, and 17-hydroxyprogesterone detected following the chromatography of extracts of unincubated adrenal glands is shown in Table I. The mobilities and staining reactions of cortisol and 11-desoxycortisol, in system E6, as well as the sulfuric acid chromogens, were identical with the designated steroids. There were insufficient amounts for further identification. No alpha-ketolic compounds whatsoever were detected in the two glands from patients with the adrenogenital syndrome. However, relatively large quantities of 17-hydroxyprogesterone were found in the adrenals from the patients with this disorder, whereas virtually none was present in beef or normal human material. The quantity of tissue extracted from the diseased organs was 2.5 Gm. (Bz) and 2.0 Gm. (Sh). In system E4, the mobility of the only detectable compound was identical with pure 17-hydroxyprogesterone: 32.5 cm. in seven hours at 23°C. It did not react with triphenyltetrazolium (TPZ) and gave a blue color with alkaline ethanolic *m*-dinitrobenzene on paper. Following elution and examination of the sulfuric acid chromogens, there was maximal absorbance at wave length 290 and 430 mμ, similar to the pure standard. The eluates from both glands were combined and oxidized with 2 per cent chromium trioxide in 90 per cent glacial acetic acid. The product revealed a single maximal absorbance in sulfuric acid at 295 mμ, as for Δ^4-androstene-3,17-dione; by infrared spectroscopy, the product was identical with the latter. In spite of diligent efforts to discover the presence

TABLE II

Conversion of 17-hydroxyprogesterone to compound F and substance S (per cent)

Source	Condition	Tissue	17-HP*	Total recovery	F†	S‡	Total conversion
		Gm.	*mg.*				
Beef	FF§	10.0	9.5	80.0	36.0‖	39.6‖	84.6
Human	FF (8 yrs.)	3.0	3.0	91.5	8.5	28.2(a)¶	36.7
Human	FF (6 yrs.)	4.0	4.0	76.5	7.5	22.3(a)	34.5
Human	PM** (3 yrs.)	2.5	2.0	83.0	4.3	6.0	10.3
Human	PM (adult)	3.0	2.5	85.0	2.4	8.6	10.0
Human	PM (adult)	2.5	2.0	86.0	2.7	13.3(b)¶	16.8
Human	PM (adult)	6.0	4.5	86.0	2.3	10.3(b)	16.9
Bz	FF†† ADH	4.5	4.5	100.0	0	0	0
Sh	PM ADH	2.0	1.6	91.0	0	0	0

* 17-HP, 17-hydroxyprogesterone (substrate).
† F, 17-hydroxycorticosterone.
‡ S, 17-hydroxy, 11-desoxycorticosterone.
§ FF, fresh frozen.
‖ Identified (infrared).
¶ a, b, identified in pairs (infrared).
** PM, postmortem.
†† ADH, congenital adrenal hyperplasia.

of 21-desoxycortisol, none was found in any of the adrenals; as little as 5 micrograms of this steroid was detectable by the methods employed, when the pure standard was chromatographed on paper. Elution of the entire paper in the zone corresponding to 21-desoxycortisol yielded no compounds reacting to the Gornall (18) reagent.

17-Hydroxyprogesterone as substrate

The results of those experiments in which 17-hydroxyprogesterone was added to the incubates are shown in Table II. The results are expressed as the percentage conversion of the substrate (based on the total amount of steroid recovered) to 11-desoxycortisol or cortisol. In all instances, including postmortem material, a significant degree of 21-hydroxylation was achieved except with the two adrenals from patients with the adrenogenital syndrome. There was usually a greater amount of 11-desoxycortisol than cortisol present, and although this might suggest that the 11-hydroxylating system deteriorated during the experiment, this is not borne out by the observations (Table III) when 11-desoxycortisol was employed as the substrate with the same tissues. It is interesting that 21-hydroxylation and 11-hydroxylation were achieved with the postmortem material, although the conversion was less than with the freshly frozen tissue. In some instances, as indicated, the identity of the acetylated products was confirmed by infrared spectroscopy. In all other cases, the criteria for identification outlined above, including the sulfuric acid chromogens, were consistent with the indicated product. Again, despite most careful scrutiny of the paper chromatograms, no 21-desoxycortisol was found in any experiment.

TABLE III

Conversion of substance S to compound F (per cent)

Source	Condition	Tissue	S*	Total recovery	F†
		Gm.	*mg.*		
Human	FF‡ (7 yrs.)	2.0	2.0	90.0	49.5
Beef	FF	2.5	2.0	96.0	69.0
Beef	FF	2.5	2.0	93.0	78.5§
Human	PM‖ (adult)	3.0	2.5	90.0	17.8
Bz	FF ADH¶	2.5	2.0	68.0	77.0(a)**
Sh	PM ADH	2.0	1.6	95.0	24.6(a)**

* S, 17-hydroxy, 11-desoxycorticosterone (substrate).
† F, 17-hydroxycorticosterone.
‡ FF, fresh frozen.
§ Identified (infrared).
‖ PM, postmortem.
¶ ADH, congenital adrenal hyperplasia.
** Identified, paired (infrared).

11-Desoxycortisol as substrate

The measurement of 11-hydroxylating activity, employing 11-desoxycortisol as the substrate, is shown in Table III. The yield of cortisol was considerably greater than when the earlier precursor, 17-hydroxyprogesterone (Table II), was employed. The diseased adrenals were capable of converting 11-desoxycortisol to cortisol with even greater facility than comparable normal glands. However, the conditions of the experiment do not allow quantitative assay of the enzymatic systems in the various trials. The ability of one diseased gland (Sh, removed after death) to accomplish this conversion suggests respectable preservation of at least the 11-hydroxylating system, lending credence to the belief that the failure of conversion when 17-hydroxyprogesterone was used as a substrate (Table II) was not due to complete deterioration.

DISCUSSION

The experiments of Hechter and Pincus (19) by perfusion of beef adrenal glands with various precursors, and of Hayano and Dorfman (10, 11) with adrenal homogenates *in vitro,* gave impetus to the studies described herein. Perusal of these contributions, previous work from this laboratory, and that of many others cited elsewhere (1–5) had suggested that in the nonhypertensive form of the adrenogenital syndrome due to congenital adrenal hyperplasia, the inability to hydroxylate the steroid nucleus at carbon 21 (deficiency of 21-hydroxylase) represented the basis of this disorder. This study gives direct evidence of a deficiency of 21-hydroxylation: Two diseased adrenal glands were unable to convert 17-hydroxyprogesterone into either 11-desoxycortisol or cortisol; a large quantity of 17-hydroxyprogesterone was initially present in each of these glands, and there were no alpha-ketolic steroids as in normal glands. The amounts of cortisol present in normal glands is slightly greater than that reported by others, but considerably more 11-desoxycortisol was found than indicated by Neher (20).

The relatively limited data have been restricted by the difficulties in obtaining fresh human adrenal tissue. It is virtually impossible to obtain tissue from patients with the adrenogenital syndrome since the treatment of this disorder has become primarily medical (21), and indeed the relative adrenocortical insufficiency renders surgery hazardous. One abnormal gland studied (Bz) was obtained under ideal circumstances; the second (Sh) is perhaps less acceptable since it was removed after death from an infant who had received small doses of cortisone. However, certain observations validate the results with the latter. The adrenal content of 17-hydroxyprogesterone of Sh was high, indicating persistent activity in this gland up to the point of 17-HP in the biosynthetic pathway. Other human postmortem tissue obtained after a similar interval of time demonstrated detectable activity with respect to both 21- and 11-hydroxylation, although this was less than with fresh material; significant 11-hydroxylation by this gland was demonstrable, although it may have been less than that obtainable under ideal conditions.

The hydroxylation of various steroid precursors by adrenal tissue *in vitro* has been described by many others, most of whom employed material from beef (9, 10, 22–34). The reports have variously indicated the presence of 11-, 17- and 21-hydroxylating systems, as well as hydroxylation at other positions. It has been shown that the "11-hydroxylase" may be concentrated in the residue and 17- and 21-hydroxylases in the supernatant (9, 27) after centrifugation. The limited amounts of tissue available precluded such fractionation here. Lombardo, Roitman and Hudson (30) have studied "normal" human adrenals and Berliner, Berliner and Dougherty (31), the glands from patients with Cushing's syndrome due to adrenal hyperplasia and adrenal carcinoma. The presence of 11-, 17- and 21-hydroxylating enzymes has been detected in these glands from the human subject.

The greater yield of 11-desoxycortisol and cortisol from 17-hydroxyprogesterone noted in these studies, as well as the failure of the diseased adrenal to convert 17-hydroxyprogesterone into detectable quantities of 21-desoxycortisol, is notable. In addition, the more effective transformation of 11-desoxycortisol into cortisol suggests that the human adrenal performs 11-hydroxylation with greater facility after 21-hydroxylation. Hayano and Dorfman have indicated virtually complete transformation of 17-hydroxyprogesterone into cortisol with beef adrenals (11); they did not indicate the presence of 11-desoxycortisol in the

course of their study. In another report, these workers (10) achieved a 95 per cent transformation of 11-desoxycortisol to cortisol by beef adrenals, an order of conversion greater than reported here. Brownie, Grant and Davidson (32) have shown that progesterone inhibits 11-hydroxylation, and that this may be overcome by the use of succinate; in view of the possibility that the substrates employed in our studies might similarly interfere with 11-hydroxylation, succinate has been added without improvement in the yield. With human adrenal tissue, Lombardo and coworkers (30) have achieved a 50 per cent conversion of 17-hydroxyprogesterone to cortisol; in these experiments, the tissue was used immediately without prior freezing. On the other hand, Berliner and associates (31) presented observations analogous to ours: In one experiment with human tissue there was a greater conversion of progesterone to desoxycorticosterone than to corticosterone and no 11-hydroxyprogesterone was detected. Thus, these workers also observed 21-hydroxylated as well as 11-, 21-dihydroxylated products. The prior occurrence of 21-hydroxylation is in accord with the conclusions of Hechter and Pincus (19). The large quantities of 11-hydroxylated 17-ketosteroids reported in the urine of affected individuals (4) remains unexplained by these studies; possibly alternative types of 11-hydroxylation occur, since 21-hydroxylation is defective in the disorder. The preponderance of 11-desoxycortisol in our incubates with 17-hydroxyprogesterone, which is unlike other work cited, may be due to technical differences in the systems such as a "suboptimal" ratio of enzyme to substrate in this study. The conditions for 11-hydroxylation by acetone-dried ox adrenal mitochondria has been well described by Grant (33).

A limited degree of activity has been detected in postmortem material in these studies. Grant, Symington and Duguid (34) failed to find any, however, when employing conditions similar to ours. The use of postmortem material is not generally advisable, and was employed here for purposes of comparison. Under these circumstances, it is especially noteworthy that specimen Sh retained its ability to hydroxylate at C-11 (but not C-21). Tissue which has been frozen at once and properly stored is satisfactory when suitable cofactors are added (10, 11, 35).

SUMMARY

The conversion of 17-hydroxyprogesterone to 11-desoxycortisol and cortisol by normal human adrenal tissue, with a greater yield of the former, has been described. The adrenal glands from two normotensive cases of the adrenogenital syndrome, due to congential adrenocortical hyperplasia, failed to effect hydroxylation of 17-hydroxyprogesterone at either carbon 11 or 21. On the other hand, when 11-desoxycortisol was employed as a substrate, both the normal and abnormal adrenal glands were able to bring about a significant conversion to cortisol. A relatively large quantity of 17-hydroxyprogesterone (but no 21-desoxycortisol) was found in the two glands from patients with the adrenogenital syndrome, without prior addition of substrate or incubation. These data directly confirm earlier indirect work pointing to a lack of "21-hydroxylase," and the presence of "11-hydroxylase" in the adrenal glands of patients with the normotensive form of this disorder. The results also suggest that 21-hydroxylation occurs prior to 11-hydroxylation in man. It has also been indicated that postmortem adrenal tissue, obtained within 24 hours of death, is capable of accomplishing the described conversions to a limited degree.

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RADIOTHYROXINE TURNOVER STUDIES IN THYROID DISEASE AFTER THERAPY

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In a previous report the disappearance curve of plasma radioactivity after intravenous injection of I^{131}-labeled l-thyroxine was employed to determine the rate of hormone degradation or removal in subjects with thyroid disease (1). Thyroxine degradation was slower than normal in untreated myxedema, and faster than normal in thyrotoxicosis. These findings were in agreement with those of Berson and Yalow (2) and Ingbar and Freinkel (3).

The present work was undertaken to observe the effects of treatment and attainment of the euthyroid state, with studies on individual subjects before and after therapy.

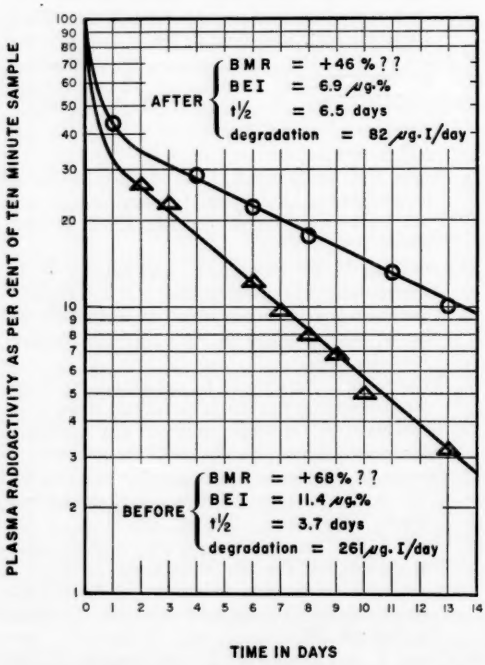

FIG. 1. RADIOTHYROXINE DISAPPEARANCE CURVE IN THYROTOXICOSIS—SUBJECT L.S., No. 13, STUDIED BEFORE AND EIGHT MONTHS AFTER I^{131} THERAPY

Semilogarithmic plot. Points after 14 days are not illustrated. Degradation rates are corrected to 1.73 M.[2] surface area. The accuracy of basal metabolism values is uncertain.

MATERIAL AND METHODS

I^{131}-labeled l-thyroxine was obtained in 1 mc. shipments from Abbott Laboratories, Oak Ridge, Tenn. The radiothyroxine was diluted in 50 to 75 ml. sterile saline to which approximately 50 mg. Red Cross albumin had been added to prevent adsorption by vessel walls. The injections were carried out within one to two hours of the preparation of dilute solutions. The subjects received tracer amounts (approximately 50 μc. in 2 to 10 μg. thyroxine) by intravenous injection. Heparinized venous blood samples were taken 10 minutes after injection and daily or on alternate days for two weeks or longer. The plasma radioactivity was assayed in a well-type scintillation counter which recorded approximately one million counts per minute (cpm) per microcurie of I^{131} (42 per cent overall efficiency) above a background of 130 cpm. Corrections for radioactive decay were made when necessary. Usually all plasma samples from a given patient and diluted aliquots of the administered compound were counted together after conclusion of the study, obviating the need for decay correction.

In all subjects two or more plasma protein bound iodine (PBI) or butanol extractable iodine (BEI) determinations were carried out[1] (4, 5). The BEI was done to avoid interference from previously administered inorganic iodine. The two determinations were used interchangeably in the calculations of "organic iodine" figures; PBI ordinarily exceeds BEI by approximately 0.6 μg. per cent in the absence of inorganic iodine (5).

Two or more basal metabolic rate (BMR) determinations were performed on separate days, usually during the period of study.

Iodine prefeeding was employed to prevent reutiliza-

[1] Performed by Bio-Science Laboratories, Los Angeles, Calif.

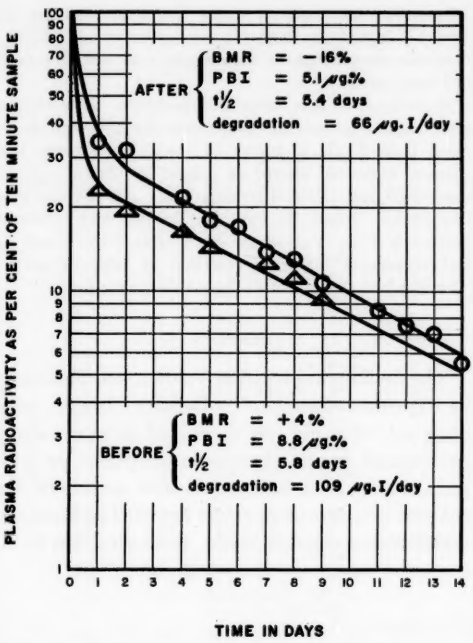

FIG. 2. RADIOTHYROXINE DISAPPEARANCE CURVE IN THYROTOXICOSIS—SUBJECT M.O., NO. 12, STUDIED BEFORE AND 15 MONTHS AFTER I^{131} THERAPY

Semilogarithmic plot. Points after 14 days are not illustrated. Degradation rates are corrected to 1.73 M.² surface area.

tion of the I^{131}-label in the thyrotoxic subjects only. Both treated and untreated thyrotoxic subjects received Lugol's solution, 15 drops daily, prior to and throughout studies. The myxedematous and normal control subjects did not receive iodine prefeeding since previous studies (1, 3) indicated that the error would be negligible except in hyperthyroid subjects.

Calculations. The injection of radiothyroxine was followed by a relatively rapid fall in the radioactivity of the plasma (Figures 1 through 4). This was attributed to the diffusion of the tracer out of the plasma throughout the body's extrathyroidal organic iodine (EOI) pool. After two days the plasma radioactivity declined more gradually, forming a straight line when plotted semilogarithmically (Figures 1 through 4). This slow exponential component of the disappearance curve was interpreted as metabolic degradation of the administered radiothyroxine, hence a measure of the turnover rate of the hormone. The computations employed have been described in detail previously (1). The half-time of thyroxine turnover was obtained graphically from the linear component of the disappearance curve. The turnover rate was computed from the half-time by the equation $k = \ln 2/t_{1/2}$, where $t_{1/2}$ represents the half-time in days, and k, the turnover rate as the fraction of the body's extrathyroidal organic iodine (EOI) pool synthesized and degraded daily. The turnover rate, k, was conveniently expressed as per cent per day.

The EOI pool was estimated from the quotient:

$$\frac{\text{Total radioactivity injected}}{\text{Radioactivity per microgram of PBI}}$$

The denominator was obtained from the zero time extrapolation which represented plasma radioactivity if distribution had occurred instantaneously after injection.

The product of the EOI pool and the turnover rate (k) gave the degradation rate in micrograms of organic iodine per day. The rates of degradation and formation should be the same in a steady state, which was assumed to exist in the absence of significant alterations in the clinical condition. The term "degradation rate" signifies rate of removal of thyroxine from the extrathyroidal pool without specification of precise metabolic pathway or route.

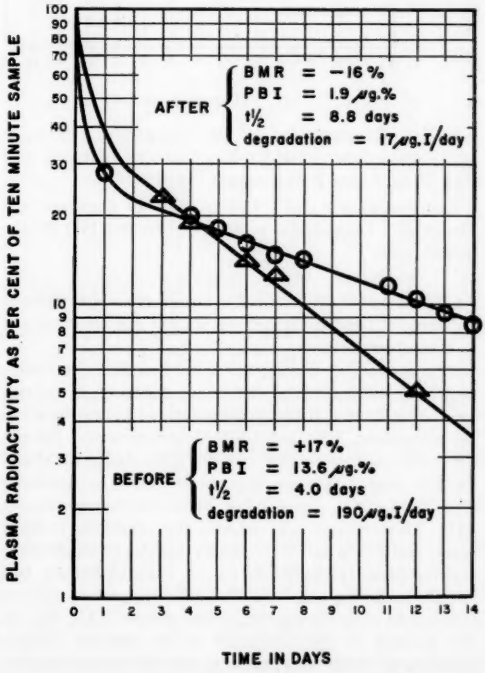

FIG. 3. RADIOTHYROXINE DISAPPEARANCE CURVE IN THYROTOXICOSIS—SUBJECT R.D., NO. 11, STUDIED BEFORE AND ONE YEAR AFTER I^{131} THERAPY

Semilogarithmic plot. Points after 14 days are not illustrated. Degradation rates are corrected to 1.73 M.² surface area.

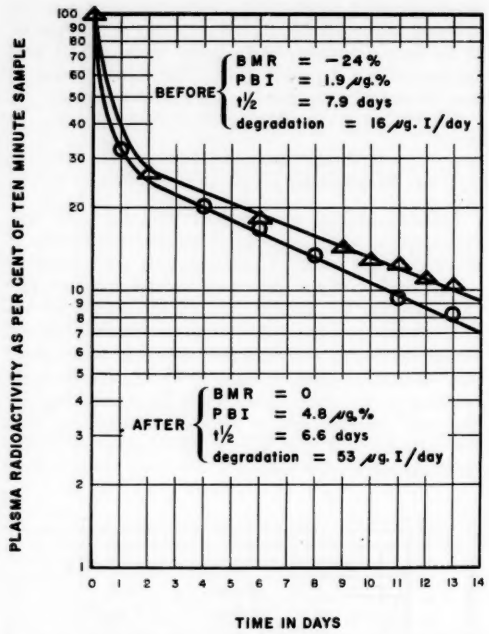

FIG. 4. RADIOTHYROXINE DISAPPEARANCE CURVE IN MYXEDEMA—SUBJECT H.L., NO. 1, STUDIED BEFORE AND ONE YEAR AFTER REPLACEMENT THERAPY

Semilogarithmic plot. Points after 14 days are not illustrated. Degradation rates are corrected to 1.73 $M.^2$ surface area.

Clinical material. The following groups were studied:

Normal. Ten healthy medical student volunteers served as control subjects.

Thyrotoxicosis. Twenty cases were studied after treatment and attainment of euthyroid status, or in certain specified instances, hypothyroid status, as judged by clinical appearance, PBI and BMR determinations. No patients with evident residual toxicity were chosen for study. Of this group six were also studied prior to treatment while still toxic, five having been reported previously (1). The treatment was subtotal thyroidectomy in three cases, and I^{131} in the rest, usually supplemented by antithyroid drug (propylthiouracil or Tapazole®) for two months beginning one week after I^{131}. No patient received antithyroid drug during the studies except M.O., No. 12. All patients of the thyrotoxic group received Lugol's solution, 15 drops daily, prior to and throughout studies, with the exception of M.O., No. 12, who received antithyroid drugs during the initial study, as described subsequently.

The diagnosis was diffuse toxic goiter (Graves' disease) in all cases except the following:

nodular toxic goiter in Nos. 12, 22, 23, 25 and 27 of Table I;

recurrent toxic goiter, status p.o. thyroidectomy, in Nos. 18, 20 and 26; and

struma lymphomatosa (Hashimoto) in No. 10, verified histologically.

Myxedema. Eight cases of myxedema were studied after various periods of replacement therapy with desiccated thyroid which, except in specified instances, had restored euthyroid status, as judged by clinical appearance, PBI and BMR determinations. All but one case had studies before therapy, five having been reported previously (1). Two of the eight cases (Nos. 2 and 7) had myxedema following resection of nontoxic goiter. The rest had spontaneous idiopathic myxedema.

RESULTS

The findings are listed in Table I, and illustrated in Figures 1 through 5. Definite changes were observed following treatment, and were especially pronounced in the thyrotoxic subjects. In subjects studied both before and after treatment, the changes in turnover rates are depicted in Figure 5, which shows changes in the same direction in all instances except one in each group.

Thyrotoxicosis

Figure 1 was selected to demonstrate the marked slowing of disappearance rate which was observed after treatment of thyrotoxicosis. The very short half-time had returned to the normal range. All treated thyrotoxic subjects except M.O., No. 12 (Figure 2), had slowing to a normal disappearance curve (Figure 1) or even slower than normal (Figure 3).

The exceptional case, M.O., No. 12 (Figure 2), showed no appreciable change in disappearance curve after I^{131} treatment and attainment of the euthyroid state. The disappearance rate was slightly accelerated in the initial and final studies. The patient had been maintained nearly euthyroid with antithyroid medications begun three weeks before and continued during the initial study (Tapazole®, 40 mg. daily; later propylthiouracil, 400 mg. daily). Although the disappearance curve remained the same, the pool size and degradation rate fell markedly after I^{131} treatment (Table I and Figure 2) in a study carried out during the administration of Lugol's solution.

In Subject L.S., No. 13, illustrated in Figure 1, despite the marked slowing of disappearance rate there was still some elevation of the pool size and degradation rate (Table I).

TABLE I

Data from radiothyroxine disappearance curves including values adjusted to 1.73 M.² surface area and 70 Kg. body weight

Condition	Subject	Sex	Age yrs.	Wt. Kg.	Surface area M.²	Therapy*	BMR†	PBI µg. %	Kinetic and isotope dilution data: Kinetics of disappearance: Half-time $t_{\frac{1}{2}}$ days	Kinetics of disappearance: Turnover rate k %/day	Extrathyroidal organic iodine pool (EOI): µg.	EOI: µg./1.73 M.²	EOI: µg./70 Kg.	Degradation rate: µg./day	Degradation rate: µg./1.73 M.²/day	Degradation rate: µg./70 Kg./day
Myxedema	H. L. 1	F	40	103.6	2.12	0	−24	1.9	7.9	8.8	233	191	158	20	16	14
			41	104.6	2.13	180 mg., 13 mos.	0	4.8	6.6	10.5	619	503	414	65	53	43
	M. A. 2	F	58	43.3	1.44	0	?−28	2.0	9.7	7.1	161	193	261	12	14	19
			59	39.1	1.37	90 mg., 6 mos.	?+15	4.8	7.4	9.4	413	522	739	39	49	70
	M. M. 3	F	58	89.0	2.03	0	−49	‡	10.3	6.7						
			59	93.6	2.06	180 mg., 14 mos.	+ 6	5.3	8.9	7.8	774	650	579	60	50	45
	C. L. 4	M	74	69.5	1.79	0	−47	2.2	12.6	5.5	292	283	295	16	16	16
			76	68.9	1.79	60 mg., 15 mos.		2.9	9.4	7.4	298	288	303	22	21	22
	M. R. 5	F	57	74.8	1.78	45 mg., 5 mos.	−14	2.4	6.5	10.7	218	212	204	23	22	22
			57	74.8	1.76	120 mg., 1 mo.	+ 4	4.9	5.6	13.1	458	450	429	57	56	53
	H. R. 6	F	61	63.2	1.66	0	−42	1.0	9.3	7.5	141	146	157	11	11	12
			62	46.4	1.47	0		2.4§	9.4	7.4	233	274	352	17	20	26
			63	49.1	1.50	60 mg., 15 mos.	−25	4.2§	8.4	8.2	379	437	540	31	36	44
	E. W. 7	F	74	68.9	1.73	0	−22	2.1	9.3	7.5	228	228	232	17	17	17
			74	68.0	1.72	120 mg., 1 mo.	− 4	4.8	10.4	6.7	485	488	499	33	33	34
	C. S. 8	F	58	83.2	1.90	120 mg., 6 mos.	− 6	3.8	6.2	11.2	392	357	330	44	40	37
Thyrotoxicosis	S. H. 9	F	44	50.8	1.50	1st I-131, 7 mos.	+10	10.1§	6.3	11.0	916	1,056	1,262	101	116	139
			44	50.8	1.50	2nd I-131, 3 mos.	−17	4.2	9.8	7.1	253	292	349	18	25	21
	L. D. 10	F	17	52.3	1.52	0		14.6	5.2	13.7	954	1,088	1,278	127	145	170
			20	58.0	1.58	Surg., 1 mo.	−19	3.6§	6.2	11.2	266	291	321	30	33	36
	R. D. 11	F	31	51.8	1.54	0	+17	13.6	4.0	17.3	974	1,094	1,315	169	190	228
			31	60.8	1.66	I-131, 12 mos.	−16	1.9	8.8	7.9	204	213	235	16	17	18
	M. O. 12	F	54	56.8	1.60	Drug, 3 weeks	+ 4	8.8	5.8	11.9	845	913	1,039	101	109	124
			55	54.5	1.58	I-131, 15 mos.	−16	5.1	5.4	12.8	472	517	606	60	66	77
	L. S. 13	F	29	54.6	1.52	0	?+68	11.4§	3.7	18.8	1,222	1,393	1,564	229	261	293
			30	74.5	1.78	I-131, 8 mos.	?+46	6.9§	6.5	10.7	790	768	742	84	82	79
	M. B. 14	F	47	42.3	1.37	0	+32	13.6	4.7	14.7	880	1,109	1,461	129	163	214
			49	51.8	1.50	I-131, 14 mos.	−26	6.2§	8.2	8.5	533	615	720	45	52	61
	R. M. 15	M	34	61.4	1.76	I-131, 10 mos.		4.1§	9.0	7.7	350	344	399	27	27	31
	T. D. 16	F	23	72.7	1.80	I-131. 14 mos.	−16	4.9§	6.4	10.8	726	698	699	78	75	75
	E. C. 17	F	54	60.8	1.63	I-131, 3 mos.	−34	2.2§	12.0	5.8	208	221	239	12	13	14
	E. P. 18	F	48	63.5	1.72	I-131, 16 mos.	−15	4.1§	9.0	7.7	262	264	289	20	20	22

* The patients with myxedema, except M. R., No. 5, had no replacement therapy prior to initial studies (designated "0") for at least three months. Cases C. L. (No. 4), H. R. (No. 6), and E. W. (No. 7) were previously untreated. H. R. was restudied a second time after omission of therapy for four months. In the studies after treatment, the final dose of desiccated thyroid and the duration on that dose is given without note of preceding lesser doses.

The initial studies on thyrotoxic patients designated "0" indicate no therapy other than Lugol's solution. S. H., No. 9, was toxic after one dose of I-131, and M. O., No. 12, was not fully controlled after three weeks of therapy with Tapazole®, later propylthiouracil. The periods after I-131 therapy refer to the time after the final dose when more than one was administered.

† BMR values represent the lowest of two or more determinations in most instances. Discrepant or uncertain determinations are indicated by a question mark.

‡ Falsely high PBI, attributed to contamination, prevented calculations from kinetic data.

§ Butanol extractable iodine (BEI) determinations performed rather than PBI determinations to avoid interference from previously administered inorganic iodine.

TABLE I—*Continued*

Condition	Subject	Sex	Age yrs.	Wt. Kg.	Surface area M.²	Therapy*	BMR†	PBI μg. %	Kinetic and isotope dilution data: Kinetics of disappearance: Half-time t½ days	Kinetics of disappearance: Turnover rate k %/day	Extrathyroidal organic iodine pool (EOI): μg.	EOI: μg./1.73 M.²	EOI: μg./70 Kg.	Degradation rate: μg./day	Degradation rate: μg./1.73 M.²/day	Degradation rate: μg./70 Kg./day
Thyrotoxicosis	P. S. 19	M	38	75.5	2.00	1-131, 13 mos.	+ 4	6.7	6.7	10.3	929	804	861	96	83	89
	J. C. 20	F	33	53.6	1.60	Surg., 12 yrs.	−15	1.9§	8.3	8.3	169	183	221	14	15	18
	H. A. 21	M	48	83.6	1.94	I-131, 6 mos.	−15	3.6	7.3	9.5	337	301	282	32	29	27
	A. Z. 22	F	55	71.8	1.64	I-131, 19 mos.	− 7	5.5	7.6	9.1	429	453	418	39	41	38
	R. R. 23	F	39	57.3	1.66	I-131, 4 mos.	+ 4	4.8§	7.8	8.9	418	436	511	37	39	45
	O. S. 24	F	43	72.7	1.90	I-131, 14 mos.	−16	5.2§	6.9	10.0	535	487	515	54	49	52
	D. B. 25	F	48	53.2	1.46	Surg., 2 mos.	?+25	6.4§	7.0	9.9	649	769	854	64	76	84
	A. V. 26	M	59	72.7	1.80	I-131, 13 mos.	+12	6.1§	7.0	9.9	557	535	536	55	53	53
	P. L. 27	F	51	50.9	1.51	I-131, 7 mos.	+ 5	5.5§	7.0	9.9	412	472	567	41	47	56
	L. T. 28	M	55	75.7	1.85	I-131, 5 mos.	− 8	5.3	7.6	9.1	647	605	598	59	55	55
Normal	Mean of 10 subjects								6.6	10.7	557	501	521	59	53	55
	Standard deviation								0.7	1.1	99	84	87	11	9	9
	Standard error of mean								0.2	0.4	31	27	28	3.3	2.9	2.9

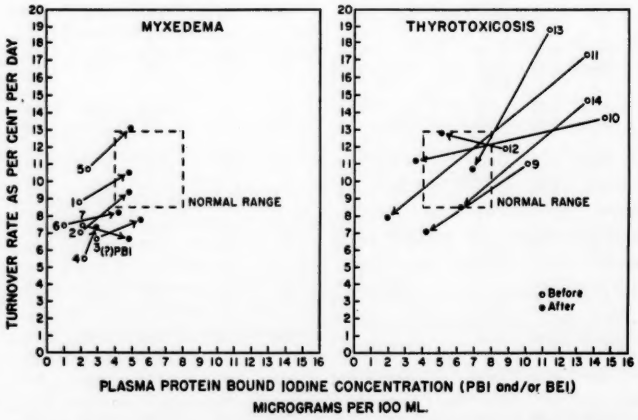

FIG. 5. RELATION BETWEEN THYROXINE TURNOVER RATE AND PBI BEFORE AND AFTER TREATMENT

The normal PBI range was taken as 4 to 8 micrograms per 100 ml. The normal range of turnover rates (8.5 to 12.9 per cent per day) was obtained from the normal mean (10.7 ± 1.1 per cent per day), plus and minus two standard deviations. The rate of fractional turnover was selected as the ordinate since this is a purely kinetic parameter computed independently of the PBI value. It will be noted that all changes in each group are in the same direction except No. 7 (E.W.) in the myxedema group, and No. 12 (M.O.) in the thyrotoxic group.

This was also the case in subject P.S., No. 19 (Table I), who had a normal turnover rate but large pool size and degradation rate. With the exceptions of M.O. (No. 12), L.S. (No. 13) and P.S. (No. 19), the remaining 17 subjects all showed reductions in turnover rate, pool size and degradation rate either to the normal range or below it.

Figure 3 was selected to illustrate an instance of a very slow turnover following treatment. Subject R.D., No. 11, was clinically hypothyroid, and the PBI, turnover kinetics, pool size and degradation rate were all within the range seen in spontaneously occurring myxedema (Table I). This was also true of Subject E.C., No. 17, who appeared clinically hypothyroid, and Subject J.C., No. 20, who appeared euthyroid 12 years after thyroidectomy, despite a low PBI value. Subjects R.M. (No. 15), E.P. (No. 18) and S.H. (No. 9) also had low kinetics, pool sizes and degradation rates but with normal PBI values and euthyroid clinical appearances.

The time interval after treatment varied. Subject L.D., No. 10, was restudied only one month after subtotal thyroidectomy and revealed marked alterations, illustrating the rapidity with which such changes could occur.

Myxedema

In contrast, the changes were slower to appear in the myxedematous patients on replacement therapy. In general the curves and values were found to have reached or approached the normal range only after considerable periods on replacement therapy. Thus, Subject H.L., No. 1 (Figure 4 and Table I), showed change to essentially normal kinetic, pool and degradation values after therapy for a year. Subject M.A., No. 2, likewise reverted to normal values after a year's treatment which consisted of 90 mg. desiccated thyroid daily during the final six months. In contrast, Subject E. W., No. 7 (Table I), showed an even slower disappearance rate when restudied after four months on desiccated thyroid (one month on 120 mg. daily); although the PBI and pool size had reached normal, the degradation rate was still low.

Subject M.R., No. 5, was studied initially on incomplete replacement therapy. When restudied after five months on increasing doses of desiccated thyroid (one month on 120 mg. daily) there was acceleration of turnover and increase to normal pool and degradation values.

Subject C.L., No. 4, was maintained at a hypothyroid level because of a myocardial infarction. After 15 months of desiccated thyroid (60 mg. daily) there was a definite acceleration of turnover, although still in the hypothyroid range. The pool size was unchanged but the degradation was increased due to the accelerated turnover.

Subject C.S., No. 8, showed all values within the normal range. Subject M.M., No. 3, had a low turnover but normal degradation rate by virtue of a high normal pool.

DISCUSSION

In the previous study (1) the metabolism of I^{131}-labeled l-thyroxine was strikingly different from normal in the hyper- and hypothyroid states. These differences were evident in the turnover rates as per cent per day, the pools of extrathyroidal hormone, and the degradation rates which represented products of pools and turnovers. All parameters were higher than normal in thyrotoxicosis, and lower in myxedema.

The present study showed quite marked changes after treatment, especially in the thyrotoxic subjects. Reversion to the normal range was seen as early as one month after subtotal thyroidectomy. Nineteen of the 20 thyrotoxic subjects had post-therapy values approximating the normal range or below it, in three instances duplicating the findings seen in spontaneous myxedema.

The exceptional patient M.O., No. 12 (Figure 2), showed a slightly accelerated turnover rate both in an initial study while maintained nearly euthyroid on drugs, and in the final study. Although the disappearance curves were essentially the same, the pool size and degradation rate became much smaller. Determination of thyroxine-binding protein (TBP) through the courtesy of Dr. Jacob Robbins revealed no abnormality in this patient's serum. With the exception of this one instance, the present findings of marked changes after therapy of thyrotoxicosis did not agree with the preliminary report of Ingbar and Freinkel (6) who observed accelerated turnovers in most patients after therapy.

Although myxedematous subjects on replacement therapy did reveal changes to or toward

the normal range, these changes occurred more slowly than with treatment of thyrotoxicosis. The previous report (1) demonstrated that rapid massive replacement therapy with triiodothyronine and thyroxine failed to alter disappearance curves in studies continued 7 to 10 days after treatment. Thus, there was no change in fractional turnover rate despite increased *absolute* amount of hormone removed with elevation of PBI concentration. Subsequent similar studies (7) continued as long as one month showed no change in plasma disappearance curves, nor in urinary excretion of the radioactive label. Moreover, these findings have been confirmed and extended by Richards, Freinkel and Ingbar (8). It is therefore quite evident that the turnover curves of myxedematous subjects are changed only by relatively prolonged replacement therapy.

In the previous report (1) it was suggested that peripheral metabolism, or some function thereof, had importance in determining hormone degradation rate. This was considered plausible in view of elevated degradation rates observed in hypermetabolic states such as acute leukemia with normal PBI concentration. Such a concept also received support from the observation by Castor and Beierwaltes (9) that dinitrophenol administration for two days, producing marked hypermetabolism, was associated with a fall in PBI concentration in normal human subjects and in an athyreotic subject maintained euthyroid on replacement therapy.

A possible mechanism is suggested by the work of Larson, Tomita and Albright (10) in which kidney slices from thyroidectomized rats converted thyroxine to triiodothyronine more slowly than slices from normal rats, while the kidneys from thyrotoxic animals (fed desiccated thyroid or exposed to cold) accomplished this conversion at an accelerated rate. The postulated adaptive enzyme system for thyroxine deiodination may well have relevance for human disappearance curve studies.

An alternative hypothesis to account for differences in hormone degradation rate pertains to the concentration of the thyroxine binding protein of plasma. The possible role of the thyroxine binding protein (TBP) was previously mentioned (1) and TBP has received increasing attention in recent work. The findings of Hamolsky, Ellison and Freedberg with regard to uptake of labeled triiodothyronine from plasma by erythrocytes (11) or diaphragm (12) may reflect variations in thyroxine bound to TBP. The studies of Albright, Larson and Deiss (13) have not suggested that binding capacity differs from normal in the hypo- or hyperthyroid state. On the other hand, Robbins and Rall, using reverse-flow electrophoresis, found the absolute TBP concentration slightly higher than normal in myxedema, but not different from normal in hyperthyroidism (14).

In any event, where plasma thyroxine concentration is higher, one might expect that the extent of "saturation" of TBP would be greater. The thyroxine not bound to TBP is "non-specifically" bound to albumin. The existence of a minute amount of "unbound" or "free" thyroxine has been postulated by Robbins and Rall based upon computations of protein association constants. Moreover, these authors have suggested that the amount of "free" thyroxine in the plasma may determine the thyroxine degradation rate, and have presented calculations compatible with such a hypothesis. On the other hand, the failure of very large doses of intravenous thyroxine to alter the turnover rate, as noted above (1), would appear to argue against such a conception. The attainment of PBI values as high as 26 micrograms per cent (1) would be expected to provide much "free" thyroxine despite possible increased thyroxine binding capacity of myxedematous plasma (14).

The data and inferences of the author, cited above, receive support from the work by Ingbar's group (8) which includes observations of displacement of thyroxine from TBP onto albumin after thyroxine loading. Despite abrupt increase of the thyroxine pool after loading, the fractional rate of turnover was unaffected. In contrast, subjects made hypermetabolic by prolonged administration of triiodothyronine showed accelerated thyroxine turnover despite reduction in PBI and computed "free" thyroxine values.

Such observations suggest that factors other than TBP must be implicated in thyroxine turnover. Recent work has underscored the need for further clarification of the nature of the binding protein or proteins. Employing starch gel electrophoresis, Rich and Bearn (15) have reported

that the first prealbumin band contained 60 per cent of the radioactivity of added radiothyroxine, and had a binding capacity far in excess of that previously reported for the interalpha zone. Ingbar (16) used a different electrophoretic method to reveal thyroxine bound in both interalpha and prealbumin areas, the latter possessing greater binding capacity, which, however, was diminished in untreated Graves' disease.

Future work will be required to settle definitely whether plasma protein binding or some rate-limiting step related to peripheral tissue metabolism is of paramount importance in determining thyroxine turnover.

A further hypothesis, embracing both of these alternatives, would assign a significant role to the turnover rate of the thyroxine binding protein itself. This would be consistent with previous evidence on serum protein metabolism, such as I^{131}-tagged albumin turnover studies which have demonstrated the accelerating effect of thyroid hormone (7, 17, 18).

SUMMARY

1. The disappearance curve of plasma radioactivity after intravenous injection of I^{131}-labeled l-thyroxine was employed to determine the rate of hormone degradation or removal.

2. Thyroxine degradation was slower than normal in untreated myxedema, and faster than normal in thyrotoxicosis.

3. In 19 of 20 cases of thyrotoxicosis treated with radioiodine or surgery, the degradation rate became approximately normal or even below normal. Pronounced changes were observed as rapidly as one month after subtotal thyroidectomy. In a single instance, the disappearance curve remained slightly accelerated after attainment of euthyroidism.

4. In two cases of postradioiodine hypothyroidism and one post thyroidectomy patient, the rate was as slow as that seen in spontaneously occurring myxedema.

5. In myxedematous patients on replacement therapy, the disappearance curve might remain unaltered for as long as one to four months despite attainment of euthyroid status. After prolonged replacement therapy of myxedema for six months or more, the degradation rates became normal or changed in the direction of normal.

6. The results suggested that the rate of removal of circulating thyroxine is a significant parameter of hormone metabolism possibly reflecting a function of peripheral tissue metabolism and/or extent of association with thyroxine-binding protein.

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CORRECTION

In the paper by Gold, Singleton, Macfarlane and Moore entitled "Quantitative Determination of the Urinary Cortisol Metabolites, 'Tetrahydro F,' 'Allo-Tetrahydro F' and 'Tetrahydro E': Effects of Adrenocorticotropin and Complex Trauma in the Human" in Volume 37, Pages 813 and 822. The chemical name for cortisol should be 11β,17α,21 trihydroxy Δ^4pregnene-3,20-dione, rather than 11β,17α,21 trihydroxy-pregnane-3,20 dione; and that for cortisone should be 17α,21 dihydroxy Δ^4 pregnene-3,11,20-trione, rather than 17α,21 dihydroxy-pregnane-3,11,20 trione.

THE MINERAL CONTENT OF NORMAL HUMAN BONE[1]

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Evidence has been forthcoming from animal experimentation that the skeleton may gain or lose electrolytes under conditions of abnormal electrolyte metabolism (1). A complete evaluation of the significance of these findings in man depends upon knowledge of the composition of normal human bone. However, information regarding human bone is sparse. In 1894, Gabriel, in a very detailed study of animal bone, included two representative samples from an unstated number of specimens of humerus of man (2). Klement, in 1936, also conducted a comprehensive investigation of mineral content, but his material was limited to three samples of skull, and two each of pelvis and femur (3). In recent years, further reports of human bone composition have appeared (4–12). However, these latter observations have not been wholly satisfactory, in that the number of samples analyzed were few and the determinations were limited to two or three minerals. Furthermore, a majority of the subjects in these reports from whom bone specimens were obtained either died with disease, or specific statements concerning the condition of the subjects prior to death were not given. The largest study currently reported is that given in abstract form by Pellegrino and Farber (13). These workers analyzed tibial bone of 15 normal subjects for Ca, P, Na and K.

The present study is concerned with the composition of bone obtained at autopsy from 16 normal adults who died suddenly. A comparative analysis of skull, rib and ilium, for water, calcium, phosphorus, carbonate, nitrogen, chloride, potassium and sodium is given.

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METHODS

Thirty-seven bone specimens (skull, rib, ilium) were obtained from 16 human subjects who came to autopsy 2 to 12 hours post mortem. The subjects died suddenly and were believed to be in normal health prior to the fatal episode. The causes of death included electrocution, subarachnoid hemorrhage, stab and gunshot wounds, strangling, and head injury. No abnormal conditions other than those causing death were disclosed at necropsy. There was no evidence of bone disorder. The mean age of the subjects was 35 years (range, 23 to 62).

Samples of bone were obtained with a saw from the occipital area of the skull, the iliac crest, and the anterior third of the ribs lateral to the sternal cartilage. The bone was stripped of muscle attachments and periosteum and broken into fragments with a mallet. Marrow was removed as completely as possible by scraping.

The specimens were dried at 105° C. for 96 hours to determine the water content. It is possible that further water loss would have occurred with heating to higher temperatures (14). The dried material was then reduced to a powder by passing it through a Wiley mill, using a 20 mesh. Fat was extracted from the powdered sample in a Soxhlet apparatus with reflux for six hours each with ethyl ether and petroleum ether. Two hundred fifty to 300 mg. aliquots of the fat-free material were then dry-ashed in platinum crucibles overnight in a muffle furnace at 525° C. The ash was dissolved in 1.5 ml. of 3 N HCl. This solution was then subjected to column separation as recommended by Forbes and D'Ambruso (15).

For preparation of the columns, dried sulfonated polysterene resin (Dowex 50×12, 50 to 100 mesh) was mixed with a small amount of water for convenience of handling and introduced to a height of 45 cm. in burettes 1.2 cm. in diameter and 60 cm. in height. The columns were washed with 300 ml. of 0.7 N HCl prior to infusion of the sample. The bone ash was transferred to the resin bed in a volume of approximately 10 ml. and elution with 0.7 N HCl at a flow rate of 2.0 ± 0.5 ml. per minute was carried out. The first 100 ml. of the eluate was used for P analysis according to Fiske and Subbarow (16). The 100 to 300 ml. fraction was utilized for Na analysis. The elutriant was then changed to 5 N HCl, and the 300 to 500 ml. fraction collected for determination of Ca and K. The latter two elution fractions were evaporated over a steam bath and made to volume for flame spectrometric determination of Na, Ca and K, using a Weichselbaum-Varney photometer. The column

TABLE I

*The composition of normal skull, rib and ilium**

	No.	H_2O (wet bone)	Fat (dry bone)	Per kilogram dry, fat-free bone							
				H_2O	Ca	P	CO_3	N	Cl	K	Na
		%	%	*Gm.*	*Eq.*	*Gm.*	*Eq.*	*Gm.*	*mEq.*	*mEq.*	*mEq.*
Skull	(10)	17.6	4.2	229	12.5	111.1	1.70	48.1	28.3	16.4	291
		3.7	2.9	63	0.27	3.7	0.06	4.5	6.1	6.8	6.8
Rib	(14)	21.6	6.4	319	11.6	104.5	1.63	53.4	35.3	34.0	268
		6.4	5.3	143	0.47	4.7	0.10	3.1	9.0	11.6	8.3
Ilium	(13)	25.8	14.1	448	11.4	100.5	1.61	56.0	41.1	38.9	269
		4.0	7.3	139	0.64	5.1	0.08	3.9	8.6	9.4	10.8

* Values given are mean and standard deviation.

was washed with 300 ml. of 0.7 N HCl after each run. Repouring of the column was usually necessary after two runs. New resin was employed after six runs. Resin separation of ions was found to be essential because of spectral interference of P and Ca in determination of Na, an effect noted also by others (17–19). In contrast, no mutual interfering effects of Ca and K were found. Average recovery values from synthetic mixtures passed through the resin columns were: P, 98.6 per cent; Ca, 99.9 per cent; Na, 99.1 per cent; and K, 101.6 per cent.

Aliquots of fat-free powder were analyzed for chloride in duplicate according to Cheek and West (20), for carbonate in triplicate according to Bergstrom (21), and for total nitrogen in duplicate by the micro-Kjeldahl method.

Analysis of fat-free powder in duplicate agreed within 2 per cent for Na, 4 per cent for K, 3 per cent for Ca, 2 per cent for P, 3 per cent for Cl, and 2 per cent for nitrogen. Triplicate analyses of carbonate were made, since the differences between individual aliquots occasionally ranged to 6 per cent. Analyses of data were made according to Snedecor (22).

It is possible that postmortem changes of pH or of some other nature might alter bone composition. However, it is believed that failure of the circulation would preclude significant removal of any substances from the areas analyzed.

TABLE II

*The variance ratios (F) of means of subjects and bones**

	Subjects	Bones
Ca	1.04	9.22†
P	11.5†	113†
CO_3‡	1.12	4.37§
Na	2.14	23.7†
N	1.87	39.7†
H_2O	0.91	18.2†
K	1.19	21.2†
Cl	6.30†	23.1†

* Degrees of freedom include 5 for subjects, 2 for bones, 10 for interaction, and 18 for analytical error.
† Significant at the 1 per cent level.
‡ In regard to CO_3, triplicate analyses were made, and the degrees of freedom for error were 36.
§ Significant at the 5 per cent level.

RESULTS

In Table I are given the mean concentrations of water and minerals expressed per kilogram of fat-free solids in skull, rib and ilium. In comparison to ilium, skull was characterized by significantly greater amounts of Ca, P, CO_3 and Na, and lesser amounts of H_2O, N, Cl and K ($p < 0.02$). The concentrations in rib ranged between those of skull and ilium. These findings are probably related to the compact nature of skull as contrasted to the cancellous nature of ilium, with the specimens of rib occupying intermediate positions in density. Because the bones compared were not always obtained from the same subjects, a check of the findings was made with variance analysis in order to eliminate the effects of patient variance on the means of bones (Table II). In six subjects from whom all three bones were obtained, the same differences between skull and ilium were found except in the case of carbonate, where the concentration was only probably decreased below that of skull ($p < 0.05$). In the same analysis, no differences in concentrations of minerals between patients were found except for those of P and Cl.

The data obtained are in general agreement with those published by others. The amounts of water found are similar to those reported by Edelman, James, Baden and Moore when the large variance is considered (11). The concentrations of Ca, P and CO_3 found agree closely with those reported elsewhere (5, 9–11, 13). Despite the differences in Ca and P concentrations between bones, the molar Ca to P ratios were 1.76, 1.72

TABLE III

Human bone sodium—mean concentration (mEq. per kilogram of dry bone) and number of samples *

Author	Reference	Skeleton	Skull	Rib	Ilium	Femur	Humerus	Tibia	Vertebr
Gabriel, 1894†	(2)						251 (2)		
Klement, 1936†	(3)		159 (3)		131 (2) (pelvis)	140 (2)			
Davies, Kornberg and Wilson, 1952‡	(8)			239 (10)		300 (6)			
Edelman, James, Baden and Moore, 1954§	(11)		273 (2)	215 (4)	222 (4)	246 (5)			215 (1)
Forbes and Lewis, 1956§	(12)	309 (2)							
Pellegrino and Farber, 1958‡	(13)							248 (15)	
Present study§			291 (10)	268 (14)	269 (13)				

* Numbers in parentheses indicate numbers of samples.
† Analysis of glycol-ashed bone. Values calculated per dry bone assuming an organic phase of 25 per cent by weight (23).
‡ No statement concerning fat extraction.
§ Values expressed per fat-free dry bone.

and 1.76 for skull, rib and ilium, respectively. The N determined approximates the amounts obtained by others (5–7). The finding of a decreased N content in skull is in keeping with the observations of Baker, Butterworth and Langley who reported that compact bone had less N than cancellous bone (5). The mean chloride concentrations obtained, ranging from 28.3 to 41.1 mEq., are equivalent to those of Edelman and co-workers (11). Concerning the K content of human bone, the only information found for comparison was in the older work of Gabriel (2), Klement (3), and in the recent report of Pellegrino and Farber (13). When the values obtained by the first two authors are recalculated, assuming an organic content of 25 per cent (23) to express the contents per kilogram of dry bone, mean concentrations of 50 and 13 mEq. are obtained, respectively. The concentration of 10 mEq. found by Pellegrino and Farber in tibia is in keeping with the amount herein obtained from skull (13).

The concentrations of Na reported have been varied (Table III). Differences in sites analyzed and in methods employed are the likely sources for these variations. The higher concentration of sodium in skull than in rib or ilium is consistent with the observations of Edelman and associates, who found similar changes in grossly normal bone obtained from five adults (11). However, the actual sodium concentrations obtained by these investigators are somewhat lower than the present results. From the data in Table III it appears that the sodium concentration is higher in compact bone than in cancellous bone.

The data were further examined in efforts to clarify the relative locations in whole bone of H_2O, Cl, K and Na. If bone is considered to be composed of two major solid phases, crystalline and organic, then the Ca and N concentrations of the total fat-free solids should serve respectively as reference bases for these phases. There is no evidence that either element exists as a significant component of the other phase. In agreement with this concept is the fact that the N and Ca concentrations expressed per fat-free solids in the current study were significantly inversely related ($p < 0.02$). Accordingly, the relations of water, Cl, K and Na to these references were studied.

In Table IV are given the common regressions obtained by combining the individual regressions of the three bones. Currently it is held that a large amount of water is associated with the hydration shell, and that Cl ions penetrate this shell (24). In the present study it is apparent that the water content was significantly related positively to N and negatively to Ca. This finding would be compatible with the concept that in the bones studied, the water determined at 105° C. existed mainly as cellular and interstitial water rather than as water of the hydration shell demonstrated to be present in synthetic apatites and veal bone by Neuman, Toribara and Mulryan (25).

TABLE IV

The covariance ratios and significances of common regression slopes (three bones) *

Regression	Slope	F	t
H_2O–N	177	1.78	3.62†
K–N	14.7	2.60	4.01†
Cl–N	13.6	1.18	4.70†
Na–N	0.76	0.77	0.18
CO_3–N	−0.13	0.66	4.03†
H_2O–Ca	−147	0.40	4.06†
K–Ca	−12.7	0.20	4.81†
Cl–Ca	−5.84	1.66	2.16‡
Na–Ca	6.17	0.44	2.08‡
CO_3–Ca	2.37	0.89	2.41§

* Degrees of freedom include 2 and 31 for the F ratio, and 33 for the t test.
† Significant at the 1 per cent level.
‡ Significant at the 5 per cent level.
§ Significant at the 2 per cent level.

In similar fashion, it is shown that Cl and K were positively related to the organic phase. However, a similar relation between water, Cl, K and N could also be obtained even if water, Cl and K were predominantly present in the hydration shell. In this situation the aging of crystals, with increase in size and displacement of water, would of necessity lead to an increased Ca to N ratio and a decrease in chloride and K. In addition, it is also possible that change in proportion of the organic phase may in some way affect the amount of water and ions associated with the hydration shell and crystalline phase. Finally, the possibility of course cannot be overlooked that water, Cl and K are located in bone in such a fashion that they would not be related to either organic or crystalline phase.

Recently Neuman and Neuman (24) have questioned the advisability of applying the calculations for interstitial water and excess sodium of bone which Harrison, Darrow and Yannet first proposed (26). Perhaps in bone which not only contains apatite but an organic phase intimately related to the apatite, the concept of a chloride space has physiologic meaning. In the present study, the mean derived chloride spaces [3] in skull and rib were 239 and 297 Gm. of water per Kg. of fat-free solids, respectively. These values do not differ significantly from the mean water contents, determined by drying, of 229 and 319 Gm. In contrast, the mean water content of ilium of 448 Gm. was significantly greater than that of the 345 Gm. derived from the chloride space. These calculations would suggest that in rib and skull the water was mainly interstitial, whereas in ilium there were additional components of cellular or hydration shell water.

It is of interest that the ratios of water, Cl and K to N were lower in skull than in rib and ilium. It may be that failure to remove all marrow in rib and ilium during the initial processing accounts in part for this discrepancy. Direct analysis of marrow revealed higher ratios of water, Cl and K to N than those found in whole bone.

Although the concentrations of Na and Ca in skull were significantly greater than those of rib and ilium (Table I), the ratios of Na to Ca (mM to M) were remarkably constant, being 46.4, 46.3 and 47.5, respectively. These constant ratios support the concept that Na is predominantly related to the crystalline phase (17). However, the common regression of Na on Ca was only probably significant ($p < 0.05$). It is possible that sufficient Na was present in the organic phase to invalidate a more significant relation of Na to Ca.

[3] Derivation of the chloride space was made assuming a plasma Cl concentration of 102 mEq. per L., 90 per cent water, and a Donnan factor of 0.95.

SUMMARY

Analyses of normal human skull, rib and ilium were made for concentrations of water, Ca, P, CO_3, N, Cl, K and Na. Skull contained significantly greater amounts of Ca, P, CO_3 and Na, and lesser amounts of water, N, Cl and K than did ilium. The composition of rib was intermediate between skull and ilium. The concentrations of water, Cl and K were related in quantity to the amount of organic phase present. Sodium appeared to be related to the crystalline phase.

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SOME EFFECTS OF MAGNESIUM LOADING UPON RENAL EXCRETION OF MAGNESIUM AND CERTAIN OTHER ELECTROLYTES [1]

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Mendel and Benedict (1) injected magnesium salts into animals and found that nearly all of the magnesium could be recovered in the urine. Incidentally, they noted that magnesium injections were followed by "a noteworthy rise in the urinary output of calcium." Schwartz, Smith and Winkler (2) made some measurements of the magnesium clearance in their study of the renal excretion of sulfate by dogs. They found that the renal clearance of magnesium was always less than that of sulfate and, therefore, "presumably the excretion of a small amount of some other cation was stimulated, in order to ensure continuous electroneutrality of urine." Chloride excretion was "almost completely repressed by sulfate excretion." In control periods, in three dogs, the renal clearances of magnesium were 2, 1 and 2 ml. per minute. Following the injection of magnesium sulfate, in five dogs, the magnesium clearances ranged from 12 to 24 ml. per minute with magnesium to creatinine clearance ratios ranging from 0.293 to 0.45.

Several investigators (3–6) have since found that the magnesium clearance increases after the injection of magnesium salts, although what appears to be the only study systematically relating clearances to plasma concentrations of magnesium has been published only in abstract form (7, 8).

Magnesium clearances, which usually have been calculated as $U_{Mg}V/P_{Mg}$, are not a simple reflection of how the kidney handles this element. Copeland and Sunderman (9), as well as others whose work they review, have shown that a third or more of the plasma magnesium is bound to proteins and therefore not filtrable at the glomerulus. Willis and Sunderman (10) have published nomograms from which filtrable magnesium and magnesium concentrations in ultrafiltrates of serum may be derived from the total magnesium and protein concentrations in serum.

The present paper extends the observations reported by others by studying the effects of magnesium over a wider range of serum concentrations, over rising, constant and falling serum levels, and in subjects whose glomerular filtration rates ranged from 25 to 196 ml. per minute.

MATERIAL AND METHODS

Thirty-four women served as subjects of the study. None of the five nonpregnant women had any discernible disturbance in hydration or cardiovascular-renal function. The remaining 29 women were selected with a view to getting cases with a wide range of inulin clearances, with representatives throughout the range. These women were all pregnant and were distributed as follows: "normal", 4; preeclampsia, 14; hypertensive disease, 7; renal disease, thought to be chronic glomerulonephritis, 4. The patients with renal disease all had hypertension, proteinuria and renal impairment. The one who did not have edema, and who had not been seen here in a previous pregnancy, was in uremia and had a urea clearance of 3 ml. per minute. Another had a urea clearance of 30 ml. per minute and the remaining two had inulin clearances of 25 and 52 ml. per minute, respectively.

All patients were on an 1,800 calorie diet, which contained about 30 mEq. of sodium and 90 mEq. of potassium. However, some had been in the hospital for only a day or two while others had been in for as long as 29 days before the tests were done.

Two salts of magnesium were used, the sulfate and the acetate.

Procedures (*a*). In the six experiments with magnesium acetate, the fasting subjects were hydrated with oral water in the early morning. At about 9 a.m. a multiple-eyed catheter was placed in the bladder and an infusion of dilute inulin and sodium para-aminohippurate (PAH) in 0.9 per cent saline was started. The concentrations of inulin and PAH depended upon the anticipated levels of the clearances of these substances. Priming doses of 3 Gm. of inulin in 10 per cent solution and 0.2 Gm. of PAH in 20 per cent solution were injected through the infusion tubing. The infusion was given at the rate of 3.7 ml. per minute, using a Bow-

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man pump. After allowing 45 minutes for equilibration, three control clearance periods were run for inulin, PAH and magnesium and for the urinary excretion rates of sodium, potassium, calcium and chloride. As soon as the last control urine had been taken, 100 ml. of 10 per cent magnesium acetate [93.2 mEq. of magnesium in $Mg(C_2H_3O_2)_2 \cdot 4\ H_2O$] were added to the 350 ml. (*circa*) of remaining infusion solution and the blood and urine collections were continued at intervals of 15 to 30 minutes until all of the infusion solution had been given. The rate of magnesium infusion was about 0.8 mEq. per minute.

Venous blood samples were taken midway between urine collections and allowed to clot. At each urine collection, the bladder was washed out with 20 ml. of distilled water and blown out with 50 cc. of air. The patients lay on their backs, in low Fowler's position, throughout the tests.

In the experiments with magnesium sulfate, several variations were tried.

(*b*). After three control periods as above, 2 Gm. of magnesium sulfate (16.2 mEq. of magnesium in 10 per cent $MgSO_4 \cdot 7\ H_2O$) was injected intravenously and enough added to the infusion solution to give about 20 to 30 mEq. of magnesium per liter and an infusion rate of 70 to 100 μEq. of magnesium per minute. After allowing 30 minutes for equilibration, three more clearance periods were run. Then another 2 Gm. of magnesium sulfate was given intravenously and the sustaining solution brought to about 41 mEq. per liter, giving an infusion rate of 0.15 mEq. of magnesium per minute. Again, 30 minutes was allowed for equilibration and then three more clearance periods were run. Three patients were studied in this way, each at three plateaus of serum magnesium concentration. The total dose of magnesium sulfate, as $MgSO_4 \cdot 7\ H_2O$, amounted to about 6.5 Gm. over a period of about three hours.

(*c*). After three control periods as above, 10 Gm. of magnesium sulfate (81.2 mEq. of magnesium) in 50 per cent solution was injected intramuscularly and the observations continued for at least four hours. In this variation the serum magnesium concentrations rose for 90 to 120 minutes, were nearly constant for the next 90 to 120 minutes, then fell off. Seven patients had concurrent measurements of inulin and PAH clearances and 12 others did not.

(*d*). In six patients the serum concentrations of magnesium were raised markedly and acutely and then held nearly constant for three to four hours by injecting almost simultaneously 3 Gm. of magnesium sulfate intravenously and 10 Gm. intramuscularly (total of 105.5 mEq. magnesium). Inulin and PAH clearances were not done in this group.

Complete protocols for representative experiments are shown in Table I.

Magnesium was measured in duplicate in serum and urine by the method of Simonsen, Westover and Wertman (11); it was often necessary to dilute the urine samples, by as much as twentyfold after large doses of magnesium. Inulin was measured in duplicate or quadruplicate by the method of Roe, Epstein and Goldstein (12). Urinary chlorides were titrated by the method of Volhard and Harvey (13). Sodium and potassium were measured with a Perkin-Elmer 52C flame photometer, using an internal lithium standard. Calcium was measured in duplicate by the method of Tisdall and Kramer (13).

The magnesium clearances, as presented, are approximations because the plasma levels of magnesium were changing during many of the observations. The expression "serum magnesium" will be used henceforth, for this is what was measured. In some contexts, this expression will be equated with plasma magnesium. "Corrected" magnesium clearances are those calculated from the filtrable magnesium, as derived from the nomogram of Willis and Sunderman (10). Whether or not their nomograms can be used legitimately will be taken up in the Discussion.

RESULTS

Effect of parenteral magnesium upon urine flow and clearances of inulin and PAH

As may be seen in the last two columns of Table II, there was no consistent effect of magnesium upon the rate of urine flow or upon the clearances of inulin and PAH. The fluid intake was constant at 3.7 ml. per minute, by infusion. No comparison of the acetate and sulfate salts was attempted, because there are too few cases. The first three of the four procedures described in the Methods section are represented in the table but were not analyzed separately, again because the cases are so few. Data are shown only for the first four (sometimes three) clearance periods in order to average the same number of observations for each period.

Considering all cases together, the average changes were small. This has been the finding of previous investigators. For instance, Heller, Hammarsten and Stutzman (3), who gave magnesium sulfate to six normal young men, found an average decrease of 9.9 per cent in the PAH clearance and an average decrease of 8 per cent in the inulin clearance. Womersley (4) found no effect of magnesium sulfate upon the inulin clearance in five normal men. Etteldorf, Clayton, Tuttle and Houck (14) loaded 15 normal children with magnesium sulfate and found no significant changes in either PAH or inulin clearances. Pritchard (15) found no significant effect upon these clearances in seven women with preeclamp-

TABLE I

Representative experiments on renal effects of magnesium loading

Subject	Time	Urine flow	Inulin clearance	PAH clearance	Serum Mg	Excretions (μEq./min.)					Mg clearance
						Na	Cl	K	Ca	Mg	
	min.	*ml./min.*	*ml./min.*	*ml./min.*	*mEq./L.*						*ml./min.*
1*	0	Prime: Inulin, 3 Gm. and PAH, 0.4 Gm.									
	Mean of 3 controls	Sustaining infusion A: Inulin, 88 mg./min. and PAH, 31.5 mg./min. in 0.9% NaCl solution at 3.7 ml./min.									
	45–102	9.89	141	684	1.58	327	367	105	3.3	9.0	5.7
	103	Sustaining infusion: 93.2 mEq. Mg in 10% $Mg(C_2H_3O_2)_2 \cdot 4\ H_2O$ solution added to A									
	102–123	10.29	153	727	1.79	322	364	106	3.6	15.2	8.5
	123–144	12.57	174	724	2.64	404	490	89	12.9	84.3	31.9
	144–162	11.33	169	706	2.92	468	481	77	17.5	113.0	38.7
	162–184	10.63	173	750	3.64	534	546	80	21.6	180.0	49.5
	184–204	13.20	184	744	4.03	454	722	89	30.1	237.0	58.8
2†	0	Prime: Inulin, 3 Gm. and PAH, 0.4 Gm.									
	Mean of 3 controls	Sustaining infusion A: Inulin, 53 mg./min. and PAH, 21 mg./min. in 0.9% NaCl solution at 3.7 ml./min.									
	45–88	5.66	60	421	1.83	234	164	60	3.3	3.3	1.8
	90	Prime: 16.2 mEq. Mg in 10% $MgSO_4 \cdot 7\ H_2O$ solution Sustaining infusion B: 5.7 mEq. Mg in 10% $MgSO_4 \cdot 7\ H_2O$ solution added to A									
	122–135	5.08	55	414	3.12	237	139	33	11.2	27.1	8.7
	135–150	4.80	48	345	3.26	260	140	29	12.4	31.6	9.7
	150–167	4.70	43	306	3.38	254	161	30	14.0	30.8	9.1
	170	Prime: 16.2 mEq. Mg in 10% $MgSO_4 \cdot 7\ H_2O$ solution Sustaining infusion C: 16.2 mEq. Mg in 10% $MgSO_4 \cdot 7\ H_2O$ solution added to 400 ml. of A									
	200–211	5.45	47	311	4.82	327	233	30	27.7	78.6	16.3
	211–224	4.47	44	286	4.50	289	192	26	24.1	64.8	14.4
	224–238	4.29	46	292	4.64	302	205	29	24.7	62.2	13.4
3‡	0	Prime: Inulin, 3 Gm.									
	Mean of 3 controls	Sustaining infusion: Inulin, 88 mg./min. in 0.9% NaCl solution at 3.7 ml./min.									
	48–104	5.06	121		1.68	47	48	16	3.8	5.7	3.4
		81.2 mEq. Mg in 50% $MgSO_4 \cdot 7\ H_2O$ solution, intramuscularly									
	104–127	4.17	107		2.02§	79§	64§	14	12.7§	19.6	9.7§
	127–148	4.00	115		2.71	97	110	19	16.4	46.3	17.1
	148–172	3.08	99		3.26	99	111	18	36.4	139.5	42.8
	172–194	3.37	103		3.75	108	115	19	42.1	172.0	45.9
	195	40.6 mEq. Mg in 50% $MgSO_4 \cdot 7\ H_2O$ solution, intramuscularly									
	194–222	2.43	96		4.47	133	102	19	46.6	215.5	48.2
	222–244	2.41	107		5.00	152	136	21	64.4	317.5	63.5
4‖	Mean of 3 controls										
	0–118	1.92			1.69	74	44	13	1.1	6.1	3.6
	118–125	24.3 mEq. Mg in 10% $MgSO_4 \cdot 7\ H_2O$ solution intravenously, plus 81.2 mEq. Mg in 50% $MgSO_4 \cdot 7\ H_2O$ solution intramuscularly									
	137–192	2.00			3.75	268	154	17	10.6	202.0	53.9
	192–252	1.75			4.16	183	143	11	11.9	177.0	42.5
	252–312	2.20			4.01	152	129	14	15.8	252.0	62.8
	312–376	1.00			3.53	73	76	5	10.5	196.5	55.7
	377–380	40.6 mEq. Mg in 50% solution of $MgSO_4 \cdot 7\ H_2O$ solution intramuscularly									
	376–437	3.43			3.44	36	51	9	7.4	159.0	46.1

* Case of essential hypertension in the thirty-ninth week of gestation. Procedure (*a*), described in section on methods.

† Case of essential hypertension in the thirty-second week of gestation. Procedure (*b*), described in section on methods.

‡ Nonpregnant "normal" patient. Procedure (*c*).

§ Not used because of uncertainty as to mean serum magnesium concentration in this period.

‖ Case of severe preeclampsia in the thirty-sixth week of gestation. Procedure (*d*).

TABLE II

Rates of urine flow (V), inulin clearance (C_I) and para-aminohippurate clearance (C_{PAH}) during and after injection of magnesium salts

Group	Cases	Control	Periods during or after Mg injection				Periods with	
			1	2	3	4	Increase†	Decrease†
		ml./min.	*ml./min.*	*ml./min.*	*ml./min.*	*ml./min.*	%	%
Normal								
V	5*	4.84	4.06	3.97	3.94	4.41	25.0	75.0
C_I	5*	127	108	107	110	118	5.0	40.0
C_{PAH}	1*	689	770	602	796		66.7	33.3
Preeclampsia								
V	4	3.85	3.85	3.14	3.77	3.82	37.5	56.3
C_I	4	98	103	124	115	110	31.2	37.5
C_{PAH}	4	422	418	351	417		50.0	8.3
Hypertension								
V	5	5.32	5.05	5.73	6.33	5.62	25.0	20.0
C_I	5	89	86	92	90	86	15.0	25.0
C_{PAH}	5	357	373	552	485	517	10.0	15.0
Renal Disease								
V	2	1.21	1.34	1.31	1.43	2.33	62.5	25.0
C_I	2	40	56	51	53		50.0	50.0
C_{PAH}	2	260	348	281	280		33.3	33.3
Totals								
V	16	4.29	3.97	3.98	4.33	4.39	32.8	46.9
C_I	16	98	94	100	98		19.4	35.4
C_{PAH}	12	390	417	444	454		29.3	17.1

* One normal pregnancy.
† Changes greater than 10 per cent.

sia, who were given 4 to 6 Gm. of magnesium sulfate intravenously.

Effect of magnesium upon the uncorrected magnesium clearance

The relationship between the total serum magnesium and the uncorrected renal clearance of magnesium is shown in Figure 1. The eight patients represented here were selected as "normals" in that they gave no evidence of disturbance in hydration or cardiovascular or renal function. There was a roughly linear increase in the magnesium clearance with rising serum concentrations of magnesium, both when magnesium sulfate and when magnesium acetate were given.

Figure 2 shows the same relationship in patients selected for a wide range in the inulin clearance. One woman in the terminal stage of glomerulonephritis and azotemia, with a urea clearance of 3 ml. per minute (inulin clearance not measured), had only a slight increase in the magnesium clearance (from 1.2 to 2.8 ml. per minute) when the serum concentration was increased from 2.1 to 4.4 mEq. per liter. At each level of glomerular filtration, the magnesium clearance increased as a linear function of the serum magnesium concentration (roughly, as in Figure 1).

Ratio of "corrected" magnesium clearance to inulin clearance

The data shown in Figure 3 are from 16 patients in whom inulin and magnesium clearances were measured simultaneously. These patients were selected to cover a wide range of inulin clearances, which, in the control periods, varied from 25 to 196 ml. per minute. The "corrected" magnesium clearances, therefore, varied widely. However, calculation of the ratio of the corrected magnesium clearance to the inulin clearance brought these data to a common base and Figure 3 indicates that the effects of the two magnesium salts were about the same, that is, the serum magnesium concentration rather than the attendant anion seems to determine the renal excretion of magnesium. It

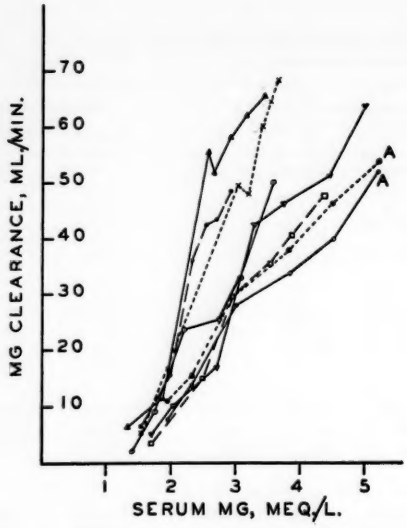

FIG. 1. THE RELATIONSHIP BETWEEN THE MAGNESIUM CLEARANCE, UNCORRECTED FOR PROTEIN BINDING OF SERUM MAGNESIUM, AND THE SERUM CONCENTRATION OF TOTAL MAGNESIUM

Eight "normal" subjects. A denotes subjects given magnesium acetate [Procedure (*a*)]; the others were given magnesium sulfate [Procedure (*c*)].

also shows that the moderately impaired kidney handled magnesium as did the normal one—that is, the tubular reabsorption or rejection of magnesium per 100 ml. of glomerular filtrate was similar from patient to patient. The triangles in Figure 3 denote observations in patients with glomerulonephritis and inulin clearances of less than 60 ml. per minute.

At the serum concentrations of magnesium studied, the clearance ratio of "filtrable" magnesium to inulin rose steeply as the serum concentration of magnesium increased. The clearance ratio approached but did not reach 1. This suggested the possibility that tubular reabsorption of magnesium might have been nearly maximal under "basal" conditions and that increments in the filtered load were excreted almost completely. Figure 4 indicates that this may have been so in 13 of the 16 patients studied. The filtered load was calculated by multiplying the inulin clearance by the concentration of magnesium in ultrafiltrate, as derived from the appropriate nomogram of Willis and Sunderman (10). In Figure 4, the increases in magnesium excretion over control values do seem to bear a 1 to 1 ratio to the increments in the calculated filtered loads, for most of the points fall close to the line describing such a relationship. Three abnormal patients (one with essential hypertension and two with preeclampsia) deviated widely from the general pattern, in that all observations fell far below the line. These patients are indicated in the graph by the broken lines connecting their points.

Tubular reabsorption of magnesium

If the nomogram of Willis and Sunderman (10) can be used legitimately for the derivation of magnesium concentrations in ultrafiltrates of plasma, Figure 4 describes the tubular reabsorption of magnesium. Points falling above the line would represent decreases in tubular reabsorption under

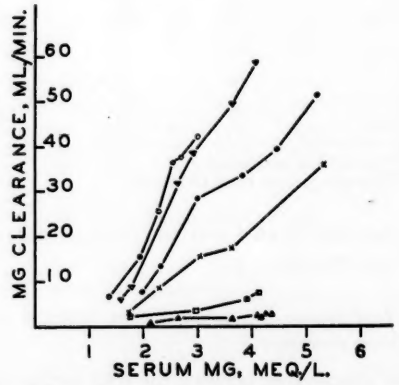

FIG. 2. THE RELATIONSHIP BETWEEN THE UNCORRECTED RENAL CLEARANCE OF MAGNESIUM AND THE SERUM CONCENTRATION OF MAGNESIUM AT VARIOUS LEVELS OF GLOMERULAR FILTRATION

Reading down from the top, the lines are as follows: ○—inulin clearance, 196 ml. per minute. Nonpregnant "normal" subject given intramuscular magnesium sulfate [Procedure (*c*)]. ▽—inulin clearance, 150 ml. per minute. Pregnant hypertensive patient given magnesium acetate infusion [Procedure (*a*)]. ●—inulin clearance, 88 ml. per minute. Nonpregnant "normal" subject given magnesium acetate infusion [Procedure (*a*)]. ×—inulin clearance, 57 ml. per minute. Pregnant hypertensive patient given magnesium acetate infusion [Procedure (*a*)]. □—inulin clearance, 25 ml. per minute. Pregnant patient with glomerulonephritis given constant infusions of magnesium sulfate [Procedure (*b*)]. △—pregnant patient in uremia. Inulin clearance not measured; urea clearance, 3 ml. per minute.

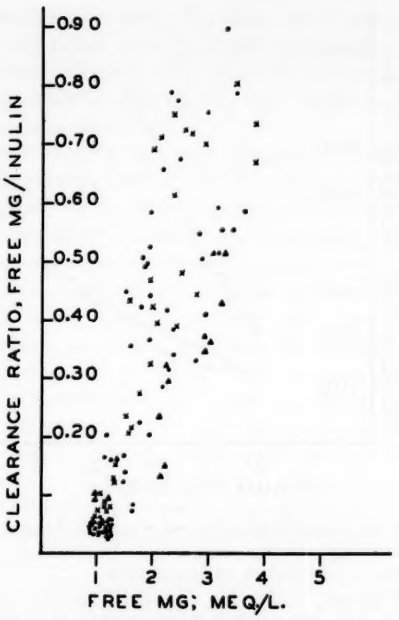

FIG. 3. THE RELATIONSHIP BETWEEN THE CLEARANCE RATIO OF "FILTRABLE" MAGNESIUM TO INULIN AND THE SERUM CONCENTRATION OF "FILTRABLE" MAGNESIUM

The inulin clearances ranged from 25 to 196 ml. per minute. ●, magnesium sulfate; ×, magnesium acetate; △, patients with glomerulonephritis.

higher filtered loads, while points below the line would represent increased tubular reabsorption. Except for the three abnormal cases, most of the points fall close enough to the line to suggest that the tubular reabsorption of magnesium was nearly constant over the range of filtered loads studied and that reabsorption was nearly maximal under "basal" conditions. When the control load was increased by less than 50 μEq. per minute, most of the points fell below the line. This would suggest that the tubular reabsorptive mechanism was not quite saturated before magnesium was injected.[2]

In the three aberrant cases the tubular reabsorption appears to have increased when the filtered load increased. In one of these, a preeclamptic, the control inulin clearance of 86 ml. per minute nearly doubled and became variable after magnesium sulfate was injected. In the other two the inulin clearance varied but little from the control values of 110 ml. per minute in the patient with preeclampsia and 170 ml. per minute in the one with essential hypertension.

Effect of magnesium administration upon calcium excretion

The usual effect of magnesium injection was to increase the urinary excretion of calcium. Roughly, the calcium excretion increased as the serum concentration of magnesium rose, as Fig-

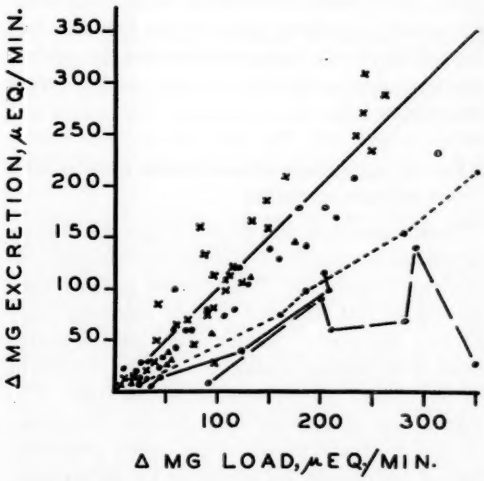

FIG. 4. THE RELATIONSHIP BETWEEN INCREMENTS IN MAGNESIUM EXCRETION AND INCREMENTS IN THE FILTERED LOAD OF MAGNESIUM

The points connected by the broken lines indicate observations in three abnormal and atypical patients. ×, normal; ●, hypertension; △, renal disease; ○, preeclampsia.

[2] In 43 observations the increments in filtered load exceeded 50 μEq. per minute. The line fitted to these points by the method of least squares had its intercept at −6, $S_a = 12.44$; this is not significantly different from a zero intercept ($t = 0.483$, $p > 0.5$). The slope of the line was 1.031, $S_b = 0.086$; this is not significantly different from a slope of 1 ($t = 0.36$, $p > 0.5$). Similarly, a line was fitted to the 23 points representing increments of less than 50 μEq. per minute in the filtered load. Its intercept was +7, $S_a = 5.4$, not significantly different from 0 ($t = 1.3$, $p = 0.2$). The slope was 0.414, $S_b = 0.2$. This is significantly different from 1 ($t = 2.93$, $p < 0.01$). Therefore the points for increments of less than 50 μEq. per minute do fall significantly below the line shown in the graph. The three aberrant cases have been excluded from this analysis.

ure 5 shows. Only seven observations were made at serum magnesium concentrations greater than 5 mEq. per liter and the average drop shown for calcium excretion at these high levels of serum magnesium probably is not real, for it was not seen in individual cases. The increases in calcium excretion following magnesium acetate were comparable to those following magnesium sulfate injections.

Calcium excretion was measured in 29 patients. Only 2 failed to show increased urinary loss of calcium after the injection of a magnesium salt. One of these had an inulin clearance of 25 ml. per minute (glomerulonephritis) and the other, with mild preeclampsia, had an inulin clearance of 110 ml. per minute. The calcium excretion in any 1 patient, other than these 2, was always maximal when the serum magnesium was at its highest level. In 12 cases, observations were made while the serum magnesium concentration first rose and later declined. In every such instance the calcium excretion decreased from its peak as the serum magnesium fell.

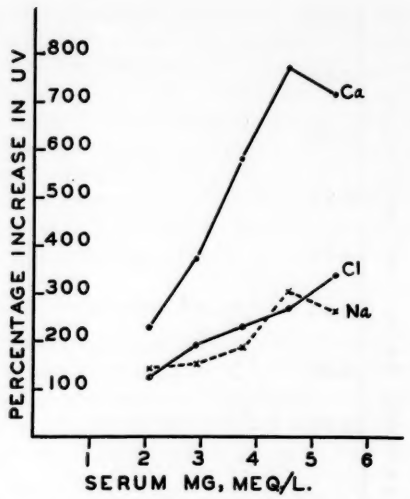

FIG. 5. THE STIMULATION OF CALCIUM, SODIUM AND CHLORIDE EXCRETIONS AND THEIR DEPENDENCE UPON SERUM CONCENTRATION OF MAGNESIUM

All patients, normal and abnormal, have been pooled. The lines connect points representing consecutive observations. Sodium and chloride excretions rose sharply while the serum magnesium was increasing, dropped precipitously while the serum magnesium was essentially constant, and fell back toward, to, or below control levels when the serum magnesium began to decline. This was observed in all six cases in which Procedure (*d*) was used. Six of the patients studied with Procedure (*c*) had serum magnesium concentrations that became constant or fell during the periods in which sodium and chloride excretions were measured; all showed the same effect. Unfortunately, inulin clearances were not measured in any of these periods and nothing can be said about a possible relationship between changing filtration rates and changes in sodium excretion.

Effect of magnesium administration upon sodium and chloride excretions

Both magnesium acetate and magnesium sulfate injections increased the sodium and chloride excretions (Figure 5). Twenty-nine patients were studied in 100 clearance periods following magnesium injections. Sodium and chloride excretions were almost equimolar and the coefficient of correlation (r) between them was 0.80. The stimulation of sodium and chloride excretions seemed to depend upon a rising serum concentration of magnesium, for whenever the serum magnesium became nearly constant or fell the excretions dropped back toward, to, or below the control levels. Only one patient failed to show an augmentation in sodium and chloride excretion while the serum magnesium was rising; she had essential hypertension and in the control periods had high excretions (chloride, 760 μEq. and sodium, 722 μEq. per minute). Following the intramuscular injection of magnesium sulfate, the excretions fell to about one-third of the control values.

Figure 6 shows what happened to sodium and chloride excretions when the serum magnesium rose, then remained nearly constant, and then fell.

Effect of magnesium administration upon potassium excretion

Four of the six patients infused with magnesium acetate had sustained decreases in potassium excretion during the infusion. The two exceptions both had depressed inulin clearances. One, with glomerulonephritis, had a control inulin clearance of 52 ml. per minute and a sustained increase in

potassium excretion. The other, with long-standing and rather severe essential hypertension, had a control inulin clearance of 57 ml. per minute and a sustained increase (of less than 10 per cent) in potassium excretion.

Following the injection, or during the infusion, of magnesium sulfate only 8 of 22 subjects had sustained decreases in potassium excretion. Six had sustained increases and these six patients were represented in the nonpregnant, normal pregnant, preeclamptic and pregnant nephritic groups.

Excluding the two patients with glomerulonephritis, in whom potassium excretions were measured, and averaging all other observations, magnesium sulfate depressed potassium excretion by a mean of 11 per cent, but the excretion was actually increased in 39 per cent of the periods. The mean decrease in potassium excretion during magnesium acetate infusions was 25 per cent and increases were seen in only 2 of 29 periods.

No correlation could be discerned between changes in potassium excretion and the serum concentrations or direction of change in serum concentrations of magnesium.

A relation was sought between changes in sodium excretion and changes in potassium excretion, on the hypothesis that magnesium might interfere with the renal tubular exchange of potassium for sodium. However, no relation was found. When sodium excretion increased, potassium excretion might decrease, increase, or remain at the same level.

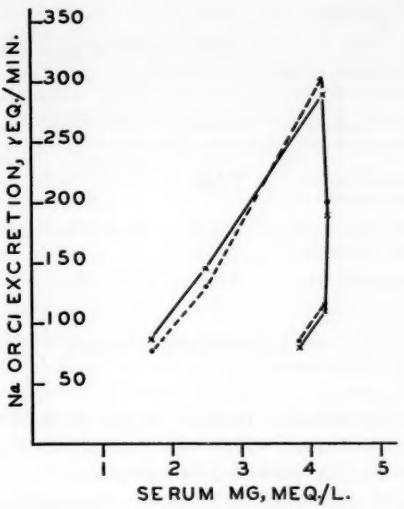

FIG. 6. SODIUM AND CHLORIDE EXCRETIONS RELATED TO THE TREND OF SERUM MAGNESIUM CONCENTRATION

The graph shows data for one representative case. The lines connect consecutive observations. Broken line denotes chloride excretion, solid line denotes sodium excretion.

DISCUSSION

The method used for the measurement of serum and urine magnesium concentrations was chosen because it gave us better recoveries of added magnesium than did the titan yellow method. Engbaek (16), citing earlier work of his own, wrote that the preliminary separation of calcium by oxalate precipitation does not interfere with the measurement of magnesium in normal serum. However, ". . . when the serum contains about 10 times the (Mg) value of the normal serum, the separation of calcium results in a loss of 4 to 8 per cent of the total magnesium." This results from the occlusion of magnesium oxalate in the calcium oxalate precipitate. This source of error was partially offset by the preliminary dilution of sera and urines expected to have high concentrations of magnesium.

Although several investigators (1–8) have shown that the renal clearance (or excretion) of magnesium increases after the injection of magnesium salts, no data have been published to show how the renal clearance of magnesium depends upon the serum magnesium concentration. (Barker, Clark and Elkinton (8) have published only an abstract of their systematic work on this.) The published clearance findings are summarized in Table III.

Figures 1, 2 and 3 indicate that the magnesium clearance increases as a roughly linear function of the serum magnesium concentration and that the attendant anion has but little effect. Magnesium acetate was chosen for comparison to the sulfate because the rapid utilization of acetate by the body would remove a large proportion of the anion, which presumably was replaced by bicarbonate. Thus the high clearance rate of such an anion as sulfate would not obligate the excretion of cations such as magnesium and a nearly "pure" effect of magnesium could be studied. Presumably the

TABLE III

Reported effects of magnesium loading upon renal excretion of magnesium

Author	Subjects	Control observations			Magnesium sulfate	After magnesium loading		
		Serum Mg	C_{Mg}*	C_{Mg}/C_I*		Serum Mg	C_{Mg}	C_{Mg}/C_I
		mEq./L.	*ml./min.*	*×100*		*mEq./L.*	*ml./min.*	*×100*
Schwartz, Smith and Winkler (2)	5 dogs		1–2		Intravenous		12–24	29–45†
Heller, Hammarsten and Stutzman (3)	6 men	1.49–1.73	3.98–6.75	2.9–5.3	1.02 mEq./min., I.V.	2.07–2.42	24–32	16–25
Womersley (4)	5 men	1.5–1.9		2.3–5.6	20 mEq., I.V.	2.3–2.8		16–22

* C_{Mg}, uncorrected magnesium clearance; C_I, inulin clearance.
† $C_{Mg}/C_{Creatinine}$.

acetate infusions resulted in the production of alkaline urines, which may have affected magnesium (and potassium) excretions.

The derivation of "filtrable" magnesium from the nomogram of Willis and Sunderman (10) assumes that the injected magnesium was promptly bound to plasma proteins to the same degree that slowly accumulated "endogenous" magnesium would be in the steady state. Hopkins, Howard and Eisenberg (17) added magnesium chloride to serum and found that in the range of 2.2 to 26.4 mg. per 100 ml. the amount of magnesium bound to proteins rose proportionately to the total serum concentration of magnesium. They found the same to be true for calcium, as did Terepka, Toribara and Dewey (18). Thus, binding sites are available and Copeland and Sunderman (9) found that magnesium proteinate acts as a dissociated salt, following the mass action law with a calculated pK of 1.77.

In the magnesium acetate infusion studies, the plasma pH may have increased somewhat. Possibly this could have affected the proportion of magnesium that was filtrable. Against this possibility is the fact that Hopkins, Howard and Eisenberg (17) found no effect of pH upon the filtrable fraction of calcium, in the range of pH 7.26 to 7.47; at higher pH levels, the filtrable fraction decreased. They also studied the effect of pH on the filtrability of magnesium, in the range of pH 7.28 to 7.46, and apparently found no effect (although this was not clearly stated so strongly).

In calculating the "corrected" clearances, as well as the filtered loads and tubular reabsorption of magnesium, the validity of the derivation of filtrable magnesium is crucial. If the injected magnesium is not bound to proteins in the same proportion as is the basal serum magnesium, then the derived data underestimate the filtered load and, therefore, the amount of magnesium reabsorbed by the tubules.

Hammarsten, Allgood and Smith (6) found that patients with advanced renal disease and azotemia had abnormally high ratios of "corrected" magnesium clearances to inulin clearances before magnesium was given. The triangles in Figure 3 show that this was true in our three cases of chronic glomerulonephritis with lesser degrees of renal impairment (inulin clearances from 25 to 60 ml. per minute). These investigators also noted that the clearance ratio did not increase so much in the nephritic patients after magnesium injection as it did in normal subjects. This is not so clear-cut in Figure 3, perhaps because of the relatively good residual function in our cases. The inulin clearance was not measured in the one patient with azotemia and a urea clearance of 3 ml. per minute, but the magnesium clearance increased only slightly after the injection of magnesium sulfate (triangles in Figure 2)—*i.e.*, the clearance ratio probably did not increase by much.

The increase in calcium excretion after the injection of magnesium salts confirms other investigations (1, 4) and in addition the present data show a good correlation between the serum concentration of magnesium and the increase in calcium loss; in individual subjects the correlation was almost linear. This was equally true for both rising and falling serum concentrations of magnesium, which is quite different from the

effect of magnesium upon the excretion of sodium and chloride. The acetate and sulfate salts were equally effective. This means that the calcium excretion was not obligated by sulfate excretion, as might be inferred from the quotation of Schwartz, Smith and Winkler (2) in the introduction. Perhaps the renal tubule cannot differentiate perfectly between calcium and magnesium, which may compete for reabsorption. No explanation is offered for the failure of magnesium to stimulate the excretion of calcium in 2 of the 29 patients studied.

The dependence of the stimulation of sodium and chloride excretions upon a rising concentration of serum magnesium explains the transitory effect noted by Womersley (4) and Pritchard (15), who gave single intravenous injections of magnesium sulfate, and the sustained effect reported by Heller, Hammarsten and Stutzman (3), who gave continuous infusions. Womersley did not find any stimulation of sodium or chloride excretion after magnesium lactate, although magnesium acetate in the present study did have such an effect. This discrepancy and the statement of Schwartz, Smith and Winkler (2) that magnesium sulfate injections almost completely repressed the excretion of chloride remain unresolved. Womersley (4) suggested that the enhanced excretion of sodium following magnesium sulfate might be obligated by the excretion of the sulfate ion. However, chloride and sodium excretions were almost equimolar in the present study (and in his own averages), and the augmentation in their excretions was as great after magnesium acetate as that following magnesium sulfate.

The stimulation of sodium excretion might be attributed to osmotic diuresis, but against this interpretation is the fact that the sodium excretion fell abruptly when the serum magnesium concentration attained a plateau (as in Figure 6) and the excretions of magnesium and calcium were essentially constant. Also, Womersley (4) did not find significant increases in the rate of excretion of total osmols following the injection of 20 mEq. of magnesium as the sulfate salt. No explanation can be offered for the peculiar observation that the sodium and chloride excretions were enhanced only so long as the serum magnesium concentration was rising.

Heller, Hammarsten and Stutzman (3), with six subjects, and Womersley (4), with five, reported significant decreases in potassium excretion after magnesium sulfate injections. Pritchard (15) found no significant change in seven women with preeclampsia. Only 12 of our 28 patients had sustained decreases, while 6 had sustained increases in potassium excretion after injection of magnesium salts.

Unfortunately, the pH of the urines was not measured but if they were alkaline after the acetate injections, as seems probable, one would expect potassium excretion to be enhanced rather than decreased. Yet the potassium excretion was decreased in 27 of the 29 periods during magnesium acetate infusion (omitting the patient with nephritis). Berliner, Kennedy and Orloff (19) have reviewed the evidence for competition between hydrogen and potassium ions for exchange with sodium ions in the tubular lumen: When the urine is alkaline, hydrogen ion secretion is suppressed and potassium secretion is augmented.

SUMMARY AND CONCLUSIONS

Some effects of parenteral magnesium acetate and sulfate upon renal excretion of electrolytes were studied in 34 "normal" and abnormal women, selected to give a wide range of inulin clearances.

The magnesium clearance increased as a roughly linear function of the serum magnesium concentration; the attendant anion—acetate or sulfate—had little or no distinguishable effect upon the excretion of magnesium.

The ratio of magnesium clearance, corrected by nomogram for protein binding of serum magnesium, to inulin clearance approached 1 at the highest levels of serum magnesium studied. Roughly, this ratio was a linear function of the serum "filtrable" magnesium.

It appears that the tubular reabsorption of magnesium may be nearly maximal under "basal" conditions. Increments in the filtered load of magnesium, over "basal" levels, are excreted almost completely. This conclusion is tentative, for there is some uncertainty as to the validity of the calculations.

The injection of magnesium salts was followed by augmented urinary excretion of calcium. The increase in calcium excretion bore a direct relation to the serum magnesium concentration.

Stimulation of sodium and chloride excretions was observed as long as the serum magnesium concentration was rising. When the serum magnesium became constant, or fell, the excretions of sodium and chloride dropped back toward, to, or below control levels.

The effect of magnesium upon potassium excretion varied from subject to subject.

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THE RELATIVE TRANSFER OF ESTROGENS AND THEIR GLUCURONIDES ACROSS THE PLACENTA IN THE GUINEA PIG[1]

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It has been suggested that estrogens can pass through the placenta because of their low molecular weight. This is supported by the demonstration of estriol in the urine of newborn male infants (1). However, there is no direct evidence to support the statment, nor is there information as to the nature of the estrogens that can or cannot be transferred.

Estrogens, in common with other steroids, are rapidly conjugated *in vivo* with glucuronic acid. This converts them into water-soluble materials, in which form they are excreted in the urine. The unconjugated estrogens appear in the urine in only trace amounts. We have used C^{14} labeled estradiol and estriol in pregnant guinea pigs, and studied the relative permeability of the placenta to the unconjugated estrogens, as well as their conjugated forms, particularly the glucuronides. In the course of the investigation, it became obvious that the customary approach to the study of placental permeability may be misleading particularly when dealing with rapidly metabolized materials, unless the effects of fetal and maternal metabolism and of placental permeability are carefully differentiated. By using a technique which excludes fetal metabolism, clearly definable results were obtained. This was accomplished at hysterotomy by clamping the umbilical cord close to the fetus and perfusing the fetal side of the placenta.

METHODS

A perfusion technique similar to one previously used in sheep (2) was adapted to the guinea pig. The fetus was delivered by hysterotomy into a saline bath maintained at 37° C., and the umbilical vessels were clamped close to the fetus. The umbilical vein and an umbilical artery were cannulated, and the fetal side of the placenta was perfused with guinea pig plasma.[3] The preparation then consisted of an artificial circulation on the fetal side of the placenta, while the maternal circulation was maintained intact. Since oxygen is supplied to the placenta from the maternal circulation, no attempt was made to oxygenate the perfusate nor were red blood cells added. The placenta was perfused at a rate of 2 ml. per minute. The placental blood flow in the guinea pig has been reported as 2.5 ml. per minute (3). With this experimental technique, the transfer rates of Na^{22}, I^{131}-antipyrine and various monosaccharides will remain constant for periods up to an hour, indicating a functioning placenta (4).

To study the transfer from mother to fetus, the estrogen dissolved in 1 ml. of plasma was injected into the maternal jugular vein and its appearance in the perfusate was noted. For fetal to maternal transfer, the estrogen was added to the perfusate, and its disappearance during two cycles of 12 ml. of perfusate was followed. In both instances the estrogen was dissolved in 0.1 ml. ethanol before adding to plasma.

Preparation of radioactive materials. Estradiol-16-C^{14} (5) and estriol-16-C^{14} (6) were synthesized in our laboratories. The radioactive impurities as indicated by chromatography on paper were about 2 per cent. The specific activity of the estradiol-17β-16-C^{14} was 9.5×10^6 counts per minute (cpm) per mg., and of the estriol-16-C^{14}, 7.0×10^6 (cpm) per mg. Estriol-16-C^{14} was synthesziedwith high specific activity (3.0×10^7 cpm per mg.) for use in Experiment 4, Table I and Experiment 3, Table II.

The glucuronides were prepared biosynthetically. The common bile duct of a guinea pig was ligated to eliminate the metabolism of the steroid that may occur through the entero-hepatic circulation. One mg. of estradiol-16-C^{14} was injected intraperitoneally and the urine was collected for 24 hours. The urine was extracted with

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[2] John and Mary Markle Scholar in Medical Science.

[3] The guinea pig plasma was diluted with two parts of normal saline in order to conserve plasma.

TABLE I

Maternal to fetal transfer of estrogens and metabolites

Injected estrogen*	Injected radioactivity	Time	Maternal radioactivity		Perfusate radioactivity	
			Ether soluble	Water soluble	Ether soluble	Water soluble
	total cpm	*min.*	*cpm/ml. plasma*		*total cpm from 0–6 minutes†*	
Free						
1. Estradiol	1.9×10^6	1	1,050	585	1,594	1,513
		6	142	459		
2. Estradiol	2.1×10^6	1	6,635	975	4,358	1,719
		6	861	993		
3. Estriol	1.8×10^6	1	2,800	650	1,664	1,018
		6	1,420	1,200		
4. Estriol	6.0×10^7	20	15,400	40,300	4,600	950
Conjugates‡						
5. Estradiol	4.3×10^5	1	14	3,600	4	36§
		6	4†	490		
6. Estradiol	4.9×10^5	1	147	5,440	11§	84
		6	15	1,210		
7. Estriol	1.3×10^6	3	758	16,800	3§	52§
		8	834	13,670		
8. Estriol		6	12	1,620	4§	100§

* Estrogens were injected into left maternal jugular vein at "0" time. One minute maternal sample was taken from right jugular and six minute sample by cardiac puncture. Fetal placenta was perfused for six minutes with 12 ml. of plasma; then total radioactivity appearing in perfusate was determined.

† Except in Experiment 4, in which estriol-16-C^{14} was given in four equal doses at 0, 5, 10 and 15 minutes and the perfusate was collected for 20 minutes.

‡ The injected conjugates were butanol extracts of urine of donor guinea pigs injected with radioactive estrogens and were 80 to 90 per cent glucuronides.

§ Calculation based on less than 6 cpm above background observed.

ether to remove unconjugated estrogens. The aqueous phase was adjusted to pH 2 and extracted with butanol. The butanol was removed *in vacuo* and replaced with water. This solution contained 98 per cent of the urinary radioactivity. To demonstrate its composition, an aliquot was incubated with β-glucuronidase (500 units per ml.)[4] at pH 5 at 37° C for four days (7), after which 92 per cent of the radioactivity was extractable with ether. No attempt was made to identify the individual components of the glucuronide fraction.

The estriol glucuronide was also prepared biosynthetically. The urine was extracted with butanol and 95 per cent of the radioactivity appeared in the extract. Eighty-seven per cent of the extract was hydrolyzable by β-glucuronidase. After incubation with β-glucuronidase, the urine was hand-extracted with ether at pH 5. The ether-soluble fraction was chromatographed on silica gel (8). All the radioactivity was eluted in the estriol fraction. Analysis by isotope dilution indicated that 85 per cent of the radioactivity was present in estriol.[5] Hence, approximately 75 per cent of the butanol extract was estriol glucuronide.

Further confirmation of the nature of the conjugates was obtained by incubating with a β-glucuronidase inhibitor and a phenolsulfatase. A solution of the estriol conjugates containing 26,000 cpm was divided into two equal parts after adjusting the pH to 5 and adding one-tenth volume of acetate buffer (pH 5). One part received 300 units per ml. of Ketodase®. In addition to the Ketodase® the second was treated with a solution of saccharic acid which had been freshly boiled at pH 3.5 for 30 minutes (9). The final concentration was 4.3×10^{-3} M with respect to saccharate. The solutions were incubated for 22 hours at 37° C. Samples were extracted with ether and the radioactivities were measured. Only 6 per cent of the radioactive conjugates was hydrolyzed in the presence of the saccharic inhibitor as compared to 76 per cent in the control. Another aliquot was incubated with sulfatase[6] (10) for 24 hours, after which time 3 per cent of the radioactivity was ex-

[4] Ketodase®, Warner-Chilcott Laboratories, N. J.

[5] The authors are indebted to Parke-Davis and Company for generous gifts of estriol.

[6] Mylase P., Wallerstein Laboratories, New York, N. Y.

TABLE II

Disappearance of estrogens from fetal perfusate *

Perfused estrogen	Initial radioactivity		Radioactivity after 1 cycle		Radioactivity after 2 cycles	
	Ether soluble	Water soluble	Ether soluble	Water soluble	Ether soluble	Water soluble
	cpm/ml.		*cpm/ml.*		*cpm/ml.*	
Unconjugated						
1. Estradiol	13,700	286	2,980	276	1,798	574
2. Estriol	5,200	208	2,755	484		
3. Estriol	107,500	4,944	46,200	3,300	19,300	3,500
Conjugate†						
4. Estradiol	16	10,000	2	9,340	25	9,890
5. Estriol	0	15,150	23	4,540	26	3,540
6. Estriol	21	10,800	14	10,000	21	10,600
7. Estriol	31	9,100	37	9,600	23	8,800
8. Estriol	22	6,600	23	5,600	19	4,600
9. Estriol	30	9,200	37	9,420	23	9,500
Conjugated and unconjugated†						
10. Estradiol	4,380	7,100	1,731	6,560	1,645	7,200
11. Estradiol	15,300	13,290	283	11,540	246	15,000

* Estrogens were added to the perfusate and samples were taken before perfusion and from the perfusion circulation (fetal side of the placenta) after 1 and 2 cycles.

† The injected conjugates were butanol extracts of urine of donor guinea pigs injected with radioactive estrogen and were 80 to 90 per cent glucuronides.

tracted with ether. A sample of estrone-16-C^{14} sulfate was quantitatively hydrolyzed under similar conditions.

Analytical techniques. After the perfusion ethanol was added to the plasma to a final concentration of 80 per cent and the mixture filtered. The protein precipitate was washed with 80 per cent ethanol and the washes were added to the filtrate. The filtrate was distilled under vacuum to near dryness and then redissolved in 10 ml. of water. This was extracted four times with 3 ml. of ether (in the estriol experiments, 20 per cent chloroform in ether was used). Samples of both phases were plated on tin-plated planchets. The radioactivity was determined in a D-46A nuclear flow gas counter and corrected to infinite thinness. The efficiency of the counter is 55 plus or minus 5 per cent.

The purity of the radioactive estrogen was determined by paper chromatography (11). Estriol was chromatographed in a solvent system of chloroform-formamide for two days and estradiol in benzene-formamide for five hours. The pattern of radioactivity was determined in a windowless automatic chromatogram scanner.[7] Nonradioactive estrogens of known purity were run concurrently either on the same paper strip or on a parallel one and the color developed by Turnbull's blue reagent (12). The purity of the radioactive sample was calculated from the amount of radioactivity found in the band corresponding to the nonradioactive estrogen.

In some experiments the radioactivity remaining in the placenta at the conclusion of the perfusion was determined. The placenta was extracted four times with 80 per cent ethanol in the Waring blendor. Less than 0.5 per cent of the radioactivity remained unextracted by this procedure.

[7] Purchased from Atomic Accessories Inc., Bellerose, N. Y.

RESULTS

I. Transfer from mother to fetus

When estradiol-C-14 was injected into the pregnant guinea pig and the fetal placenta perfused, ether-soluble materials were recovered in the perfusate (Table I). However, estradiol-C-14 could not be demonstrated in the perfusate by paper chromatography. When the experiments were repeated with estriol-C-14 unequivocal evidence of transfer of the unaltered estrogen was obtained. In Table I, Experiment 4, only one peak of radioactivity was demonstrated on paper chromatography of the ether-soluble fraction of the perfusate and this corresponded to the mobility of carrier estriol. The precipitous change in maternal blood levels of estriol that must occur while the placenta is being perfused does not permit any precise interpretation of the rate of transfer, but it is evident that transfer is rapid.

Following the injection of estriol into the

mother, the water-soluble radioactivity recovered in the perfusate was not hydrolyzable by β-glucuronidase. However, the enzyme did hydrolyze about 50 per cent of the water-soluble radioactivity found in the maternal blood at the end of the experiment. Similar observations were made with estradiol. The findings suggested placental permeability to some estrogen conjugates but not to glucuronides. This impression concerning the glucuronides was confirmed by injecting biosynthetically prepared glucuronide into the mother (Table I). Very little radioactivity was recovered in the placental perfusate. The nature of the conjugates to which the placenta is permeable is under investigation.

II. Transfer from fetus to mother

In the investigation of transfer across the placenta from fetus to mother, the radioactive estrogens were added to the perfusate and the disappearance of radioactivity after the first and second cycle was recorded. The results (Table II) clearly showed a rapid disappearance of the estrogens, a large amount during the first cycle and less in the second. In contrast, the level of glucuronides in the perfusate remained essentially unchanged after perfusion in all experiments except one.

The question arose as to whether the disappearance of radioactivity from the perfusate did, in fact, indicate transfer across the placenta. To demonstrate this, estriol with high specific activity was perfused through the placenta (Table II, Experiment 3) after which it was possible to identify estriol-C-14 in the maternal blood by paper chromatography. It would be expected that the estrogens transferred to the mother would rapidly disappear from the maternal circulation (13) so that no quantitative interpretation of transfer can be made from the amount recovered in the maternal plasma. However, indirect evidence indicates that the disappearance of radioactivity from the perfusate is primarily the result of transfer. Storage of estrogens in the placenta during the course of the perfusion was excluded by determining the radioactivity in the placenta. In Table II, Experiment 3, a total of 1.9×10^6 cpm of estriol-C-14 was added to the perfusate. At the end of two cycles the entire placenta contained only 75,000 cpm. This represents only 5 per cent of the 1.5×10^6 cpm that disappeared from the perfusate. However, the accumulation of estriol that did occur in the placenta during the perfusion reduced the gradient from the perfusate and may be responsible for the reduced rate of disappearance of estrogens during the second cycle.

The ether-soluble radioactivity remaining in the perfusate at the end of Experiment 3 (Table II) was still estriol, indicating that the placenta did not significantly metabolize this estrogen in the course of the experiment.

It would appear from the foregoing that a quantitative estimate of transfer can be made from the disappearance of radioactivity from the perfusate, and that it is very rapid.

It may be noted that roughly 5 per cent of the radioactivity in the perfusate in the first three experiments in Table II is water-soluble *before* perfusion. This is puzzling because less than 1 per cent of the radioactivity added to the perfusate was water-soluble. We have no explanation and do not know if it is an artifact of the analytical procedure or the result of interaction with the plasma. However, it does not affect interpretation of the results.

DISCUSSION

Our first attempts to study the transfer of estrogens across the placenta were done in what might be considered the orthodox manner. Estradiol-C-14 was injected intravenously into the pregnant guinea pig and because of the extremely rapid disappearance of radioactivity from the maternal blood stream, maternal and fetal blood samples were taken one minute later. The relatively large amount of water-soluble radioactivity in the fetal plasma suggested a preferential transfer of the conjugates across the placenta. However, we subsequently found that the fetus could also rapidly convert estradiol into water-soluble materials producing the erroneous impression of preferential permeability. It became evident that a technique must be used that would make it possible to separate the effects of fetal metabolism from placental transfer.

When the complicating feature of fetal metabolism was removed by replacing the fetal circulation with a perfusion system, all suggestion of preferential transfer of the conjugates disappeared.

Although maternal metabolism still limited the quantitative interpretation of the data, it was possible to demonstrate rapid transfer of estriol-C-14 in both directions. Estriol has not been demonstrated to be a normal product of guinea pig metabolism, but it proved to be useful in our studies. We were unable to demonstrate the transfer of estradiol probably because estradiol is rapidly metabolized and less radioactivity was used than in the estriol experiments. However the ether-soluble metabolites of estradiol move rapidly across the placenta in both directions.

Following the intravenous injection of radioactive estrogens into the mother, the radioactivity disappears extremely rapidly from the plasma (Table I). Less than 7,500 cpm per ml. could be detected in the maternal plasma one minute after the injection of approximately two million counts per minute into the maternal jugular vein. This cannot be explained by simple equilibration within body fluids and at present is unexplained. A similar experience has been reported in humans (13). The rapid disappearance of injected radioactivity from the maternal circulation is probably largely responsible for the fact that less than 1 per cent reaches the fetal circulation. It also prevents any precise estimate of the rate of transfer of these materials across the placenta from mother to fetus.

The experiments on transfer from fetus to mother were not complicated by a rapidly falling estrogen level. The radioactivity in the perfusate entering the placenta was maintained at a constant level and it is evident that a considerable amount of the unconjugated estrogens disappeared during the first cycle (Table II). Since only an insignificant amount was recovered in the placenta at the end of the experiment it can be assumed that the rest was transferred across the placenta. The greatly reduced transfer on the second cycle may be the result of a reduced gradient produced by the small retention of estrogens in the placenta.

These results contrast sharply with those with the glucuronide conjugates, little or none of the latter being transferred across the placenta in either direction. It has been reported that the fetal guinea pig does not form glucuronides effectively (14). This may represent a significant advantage in the disposal of materials *in utero* since the placenta is relatively impermeable to the glucuronides. Once the ether-soluble estrogens are transfererd to the maternal circulation, conjugation and renal excretion by the mother would follow.

Estrogens are transported in the plasma bound to proteins. To make certain that the estrogens in the perfusion experiments were protein-bound, in one experiment estradiol-C-14 was solubilized in plasma diluted with saline as usual and then dialyzed against saline for 72 hours at 4° C. At the end of this time, 97 per cent of the radioactivity was still retained in the cellophane bag.[8] When this material was perfused through the fetal placenta the results resembled those presented in Table II. In the solution prior to perfusion there were 9,130 cpm per ml., which dropped to 3,070 after one cycle and 2,170 after the second.

Protein-bound estrogens exchange very slowly across inert membranes (15). Since plasma proteins exchange slowly between mother and fetus (16), the very rapid transfer of estrogens across the placenta suggests a mechanism for rapidly releasing the protein-binding at the placental membrane. The investigations of physiologists suggest a possible explanation (17). It has been demonstrated that a major factor determining rate of transport is the oil to water partition of the substance under study—the more lipid-soluble, the more rapidly transported. From this, it has been deduced that a large portion of the membrane area is lipid in character. Water-soluble materials must travel through the relatively sparsely situated water-filled pores, the molecular size of the material being a major controlling factor in the rate of transport. In applying this theory to our observations, one may suspect that the greater solubility of the unconjugated estrogens in lipids could lead to extraction from the circulating fetal plasma by the placental membrane. The extraction of the unbound, unconjugated estrogens from the plasma could displace the equilibrium and rapidly release more estrogens from their protein-binding. Alternatively, the placental membrane may compete favorably with the plasma proteins for the bound estrogens. Partition of the extracted lipids could then take place with

[8] In a control experiment in which no plasma was placed in the dialysis bag, the radioactivity was distributed equally inside and outside the bag within 24 hours.

the estrogen-poor maternal plasma on the other side of the membrane. This mechanism would not be available to the water-soluble glucuronide.

At first glance, the rapid transfer of ether-soluble estrogens and the relative impermeability of the glucuronides through the placenta are difficult to reconcile with the performance of the kidney where the glucuronide is preferentially excreted. The difference in structure between the two organs suggest explanations, a detailed consideration of which is outside the scope of this paper.

SUMMARY

The transport of estrogens across the placenta was studied using the following technique: The guinea pig fetus was removed by hysterotomy and the fetal placenta perfused *in situ* leaving the maternal circulation intact. This eliminated the complicating feature of fetal metabolism. With this technique it was possible to demonstrate the rapid transfer of estriol-16-C^{14} and the ether-soluble metabolites of estradiol-16-C^{14} across the placenta in both directions. However, the placenta was relatively impermeable to the water-soluble estrogens found in urine, 80 to 90 per cent of which are glucuronides. The possible significance of protein-binding and of glucuronide conjugation in transport and excretion is briefly discussed.

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STUDIES ON KETONE METABOLISM IN MAN. II. THE EFFECT OF GLUCOSE, INSULIN, CORTISONE AND HYPOGLYCEMIA ON SPLANCHNIC KETONE PRODUCTION [1]

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We have previously described a technique which utilizes hepatic venous catheterization for the estimation of net splanchnic ketone body production (NSKP) in man (1). In a series of subjects without metabolic abnormality, a small but significant endogenous NSKP was detected in the postabsorptive state, and upon the intravenous administration of a ketone body precursor, sodium octanoate, the NSKP was increased fivefold. The present report concerns the application of this technique to the study of the effects of glucose, insulin, cortisone and hypoglycemia on ketone body production in man.

METHODS

Twenty-seven mildly ill male hospital patients between the ages of 23 and 58 years, without metabolic abnormality, were used as subjects and studied after a 12 to 14 hour fast. The criteria of selection and management of subjects, technique of hepatic venous catheterization, methods of chemical analysis, and calculations are identical to those previously reported (1). The 16 normal males in the study will serve as the control series, designated "1955 series," for the present experiments.

The subjects of the present experiments were divided into four groups and studied in a manner similar to the "1955 series" except for the specific metabolic alterations noted below for each group under "Results." The general plan of study for all groups was as follows: After mild phenobarbital sedation, inlying femoral arterial and forearm venous needles and the hepatic venous catheter were inserted. An intravenous infusion of bromsulphalein was started and maintained throughout the period of study. A control study was made on each subject during which femoral arterial and hepatic venous bloods were each sampled four times at five minute intervals for the subsequent determination of bromsulphalein, glucose and total ketone concentrations. A 500 ml. infusion of a 1.5 per cent solution of sodium octanoate was then administered intravenously at a constant rate over a one hour period, during which time blood sampling was repeated four times at 15 minute intervals. Blood sampling for oxygen content and saturation was interspersed at appropriate intervals during each period. The estimated hepatic blood flow (EHBF) was determined by the technique of Bradley, Ingelfinger, Bradley and Curry (2). The net splanchnic glucose production (NSGP) and net splanchnic ketone production (NSKP) are the products of the hepatic blood flow and the hepatic venous-arterial blood glucose and ketone differences, respectively. The net splanchnic oxygen consumption (SPO_2) was calculated by multiplying the arterial-hepatic venous blood oxygen differences by the EHBF.

In addition to the above studies, peripheral venous blood total ketones and glucose concentrations during a four hour insulin tolerance test (0.1 unit per Kg., I.V.) were estimated in 11 normal medical students with and without 200 mg. of cortisone acetate being administered orally one hour prior to the insulin.

RESULTS

1955 series

The data, including the mean plus or minus standard error and standard deviation, for the control series of 16 male subjects previously reported in detail (1), are given at the bottom of Table I. Each subject was studied first in the postabsorptive state, the *control period,* and then during the hour-long infusion of 500 ml. of 1.5 per cent sodium octanoate solution, henceforth designated the *octanoate period.*

Glucose series (Table I)

Six subjects were studied in a manner differing from the 1955 series only in that 20 Gm. of glucose was mixed with the octanoate infusion. Thus, as expected, the data of the control period were entirely comparable to those in the 1955 series of

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TABLE I

The metabolic effects of intravenously administered sodium octanoate mixed with glucose

Patient, Age	Diagnosis, Body surface area	Anxiety during test	Total ketones (*μM acetone/100 ml. blood*)								
			Control			During sodium octanoate			Δ	Δ	Δ
			Fem. art.	Hep. vein	H.V.–F.A.	Fem. art.	Hep. vein	H.V.–F.A.	Fem. art.	Hep. vein	H.V.–F.A.
yrs.											
H. M. 37	Functional G.I. 1.71	1+	22.5	30.3	7.8	58.0	80.0	22.0	+35.5	+49.7	+14.2
W. H. 28	Duodenal ulcer 1.59	1+	11.0	15.8	4.8	45.2	48.2	3.0	+34.2	+32.4	− 1.8
J. F. 31	Duodenal ulcer 1.66	1+	14.5	19.2	4.7	52.5	65.2	12.8	+38.0	+46.0	+ 8.1
H. K. 52	Duodenal ulcer 1.91	1+	13.8	15.8	2.0	34.5	51.5	17.0	+20.7	+35.7	+15.0
T. G. 28	Cervical disc 1.77	1+	9.5	11.8	2.3	27.3	36.8	9.5	+17.8	+25.0	+ 7.2
B. U. 30	Functional G.I. 1.98	1+	10.0	16.0	6.0	25.8	41.5	15.7	+15.8	+25.5	+ 9.7
Mean age, 34	Mean S.A., 1.77	Mean S.E.	13.6	18.1	4.5	40.6	53.9	13.3	+27.0	+35.8	+ 8.8
		S.D.	1.97	2.81	0.87	5.46	6.57	2.68	4.05	4.22	2.45
Range, 28–52			4.83	6.89	2.14	13.39	16.09	6.57	9.93	10.34	6.00
Statistical comparison with control series		p	>0.5	>0.5	>0.5	>0.5	>0.05 <0.1	<0.01	>0.5	>0.05 <0.1	<0.01
Previously reported control series (16 male subjects)		Mean	14.1	18.4	4.3	42.6	64.8	22.2	+28.5	+46.4	+17.9
		S.E.	1.73	2.24	0.62	2.56	2.94	1.10	2.26	2.83	1.19
		S.D.	6.92	8.96	2.46	10.25	11.79	4.40	9.01	11.31	4.77

Patient, Age	Diagnosis, Body surface area	Anxiety during test	Estimated hepatic blood flow *ml. blood/min./M.²*			Net splanchnic ketone production *μM acetone/min./M.²*			Net splanchnic oxygen consumption *ml./min./M.²*		
			Control	During sod. oct.	Δ	Control	During sod. oct.	Δ	Control	During sod. oct.	Δ
yrs.											
H. M. 37	Functional G.I. 1.71	1+	808	828	+ 20	62	182	+120	34	39	+ 5
W. H. 28	Duodenal ulcer 1.59	1+	548	556	+ 8	28	17	− 11	33	40	+ 7
J. F. 31	Duodenal ulcer 1.66	1+	740	751	+ 11	35	96	+ 61	29	32	+ 3
H. K. 52	Duodenal ulcer 1.91	1+	694	548	−146	16	88	+ 72	28	29	+ 1

TABLE I—*Continued*

Patient, Age	Diagnosis, Body surface area	Anxiety during test	Estimated hepatic blood flow *ml. blood/min./M.²* Control	During sod. oct.	Δ	Net splanchnic ketone production *μM acetone/min./M.²* Control	During sod. oct.	Δ	Net splanchnic oxygen consumption *ml./min./M.²* Control	During sod. oct.	Δ
yrs.											
T. G. 28	Cervical disc 1.77	1+	1,106	1,238	+132	28	126	+ 98	36	42	+ 6
B. U. 30	Functional G.I. 1.98	1+	764	819	+ 55	46	129	+ 83	35	46	+11
Mean age, 34	Mean	Mean	777	790	+ 13	35.8	106.3	70.5	32.5	38	+ 5.5
	S.A.,	S.E.	75.3	102.9	34.6	6.60	22.4	18.34	1.35	2.59	1.41
Range, 28–52	1.77	S.D.	184.0	252.1	84.8	16.2	54.8	44.9	3.31	6.36	3.46
Statistical comparison with control series		p	>0.5	>0.3 <0.4	>0.1 <0.2	>0.5	<0.01	>0.01 <0.02	>0.5	>0.4 <0.5	>0.5
Previously reported control series (16 male subjects)		Mean	790	732	− 58.0	34.0	158.0	+124	36.1	40.3	+ 4.2
		S.E.	38.0	40.1	27.15	5.20	6.86	7.36	2.05	1.52	1.46
		S.D.	151.7	160.4	108.6	20.86	27.46	41.4	7.94	5.83	5.66

Patient, Age	Diagnosis, Body surface area	Anxiety during test	Conversion sod. oct. to ketones	Femoral artery glucose *mg./100 ml. blood* Control	During sod. oct.	Δ	Net splanchnic glucose production *mg./min./M.²* Control	During sod. oct.	Δ	BSP clearance % Control	During sod. oct.	Δ
yrs.			%									
H. M. 37	Functional G.I. 1.71	1+	14	89	149	+60	50	−30	− 80	94	94	0
W. H. 28	Duodenal ulcer 1.59	1+	0	81	149	+68	62	− 8	− 70	85	83	−2
J. F. 31	Duodenal ulcer 1.66	1+	7	89	140	+51	65	−10	− 75	90	84	−6
H. K. 52	Duodenal ulcer 1.91	1+	10	87	131	+44	72	4	− 68	99	93	−6
T. G. 28	Cervical disc 1.77	1+	12	94	160	+66	96	−41	−137	86	78	−8
B. U. 30	Functional G.I. 1.98	1+	11	81	128	+47	64	−10	− 74	100	103	+3
Mean age, 34	Mean	Mean	9.0	86.6	143	56.4	68.2	−15.8	− 84	92.3	89.2	−3.1
	S.A.,	S.E.	2.02	2.04	4.96	4.12	6.28	6.72	10.73	2.61	3.74	1.73
Range, 28–52	1.77	S.D.	4.96	5.00	12.15	10.1	15.40	16.50	26.30	6.40	9.16	4.23
Statistical comparison with control series		p	<0.01	>0.5	<0.01	<0.01	>0.5	<0.01	<0.01	>0.1 <0.2	>0.2 <0.3	>0.5
Previously reported control series (16 male subjects)		Mean	14.9	84.6	81.6	− 3.0	68.0	65.0	− 3.0	84.4	82.2	−3.4
		S.E.	0.88	1.87	1.76	0.5	4.47	6.0	5.48	3.19	3.32	0.73
		S.D.	3.46	7.46	7.05	2.01	17.92	24.08	21.82	12.81	13.04	2.85

TABLE II

The metabolic effect of intravenously administered sodium octanoate mixed with glucose and insulin

Patient, Age	Diagnosis, Body surface area	Anxiety during test	Total ketones (μM acetone/100 ml. blood)								
			Control			During sodium octanoate			Δ	Δ	Δ
			Fem. art.	Hep. vein	H.V.–F.A.	Fem. art.	Hep. vein	H.V.–F.A.	Fem. art.	Hep. vein	H.V.–F.A.
yrs.											
C. M. 39	Cluster headache 1.72	2+	11.0	11.2	0.2	26.7	37.3	10.6	+15.7	+26.1	+10.4
A. H. 28	Duodenal ulcer 1.92	2+	13.5	17.8	4.3	33.0	44.0	11.0	+19.5	+26.2	+ 6.7
A. W. 41	Low back pain 1.76	1+	9.5	12.0	2.5	30.0	48.0	18.0	+20.5	+36.0	+15.5
P. S. 47	Peptic ulcer 1.78	2+	11.0	17.2	6.2	34.0	58.2	24.2	+23.0	+41.0	+18.0
G. F. 33	Epilepsy 1.84	1+	4.2	6.5	2.3	22.0	35.0	13.0	+17.8	+28.5	+10.7
J. G. 30	Peptic ulcer 1.87	1+	12.0	14.7	2.7	31.8	48.5	16.7	+19.8	+33.8	+14.0
C. L. 23	Arrested coccidio-mycosis 1.92	1+	8.8	14.0	5.2	24.5	38.2	13.7	+15.7	+24.2	+ 8.5
E. E. 31	Duodenal ulcer 1.75	1+	36.2	50.8	14.6	52.0	79.5	27.5	+15.8	+28.7	+12.9
Mean age, 34	Mean S.A., 1.82	Mean S.E.	13.3	18.0	4.7	31.8	48.6	16.8	+18.5	+30.6	+12.1
		S.D.	3.41	4.85	1.54	3.25	5.15	2.18	1.04	1.98	1.31
Range, 23–47			9.66	13.72	4.37	9.20	14.58	6.16	2.94	5.61	3.72
Statistical comparison with control series			>0.5	>0.5	>0.5	>0.02 <0.05	<0.01	>0.02 <0.05	<0.01	<0.01	<0.01

Patient, Age	Diagnosis, Body surface area	Anxiety during test	Estimated hepatic blood flow ml. blood/min./M.²			Net splanchnic ketone production μM acetone/min./M.²			Net splanchnic oxygen consumption ml./min./M.²		
			Control	During sod. oct.	Δ	Control	During sod. oct.	Δ	Control	During sod. oct.	Δ
yrs.											
C. M. 39	Cluster headache 1.72	2+	731	766	+ 35	3	81	+ 78	34	42	+ 8
A. H. 28	Duodenal ulcer 1.92	2+	1,088	1,147	+ 59	46	132	+ 86	31	38	+ 7
A. W. 41	Low back pain 1.76	1+	623	574	− 49	16	100	+ 84	33	33	0
P. S. 47	Peptic ulcer 1.78	2+	820	689	−131	52	167	+115	33	34	+ 1

TABLE II—*Continued*

Patient, Age	Diagnosis, Body surface area	Anxiety during test	Estimated hepatic blood flow *ml. blood/min./M.²* Control	During sod. oct.	Δ	Net splanchnic ketone production *μM acetone/min./M.²* Control	During sod. oct.	Δ	Net splanchnic oxygen consumption *ml./min./M.²* Control	During sod. oct.	Δ
yrs.											
G. F. 33	Epilepsy 1.84	1+	627	632	+ 5	14	82	+ 68	28	38	+10
J. G. 30	Peptic ulcer 1.87	1+	631	633	+ 2	18	106	+ 88	25	34	+ 9
C. L. 23	Arrested coccidio-mycosis 1.92	1+	678	775	+ 97	34	108	+ 74	38	43	+ 5
E. E. 31	Duodenal ulcer 1.75	1+	763	815	+ 52	110	223	+113	37	45	+ 8
Mean age, 34	Mean	Mean	745	754	+ 9	36.6	125.0	+ 88.4	32.4	38.3	+ 5.9
	S.A.,	S.E.	54.8	63.25	23.70	12.06	17.14	6.07	1.54	1.59	1.31
Range, 23–47	1.82	S.D.	155.0	179.0	67.09	34.12	48.52	17.19	4.36	4.49	3.73
Statistical comparison with control series				>0.5	>0.5	>0.1 <0.2	>0.5	>0.02 <0.05	<0.01	>0.2 <0.3	>0.3 <0.4	>0.4 <0.5

Patient, Age	Diagnosis, Body surface area	Anxiety during test	Con-version sod. oct. to ketones	Femoral artery glucose *mg./100 ml. blood* Control	During sod. oct.	Δ	Net splanchnic glucose production *mg./min./M.²* Control	During sod. oct.	Δ	BSP clearance % Control	During sod. oct.	Δ
yrs.			%									
C. M. 39	Cluster headache 1.72	2+	9	80	106	+26	55	− 7	− 62	79	72	−7
A. H. 28	Duodenal ulcer 1.92	2+	11	86	113	+27	73	+13	− 60	96	91	−5
A. W. 41	Low back pain 1.76	1+	10	82	93	+11	−1	− 2	− 1	70	61	−9
P. S. 47	Peptic ulcer 1.78	2+	14	80	102	+22	58	+ 4	− 54	91	90	−1
G. F. 33	Epilepsy 1.84	1+	8	82	94	+12	91	16	− 75	84	84	0
J. G. 30	Peptic ulcer 1.87	1+	11	82	111	+29	136	+ 2	−134	74	65	−9
C. L. 23	Arrested coccidio-mycosis 1.92	1+	9	79	111	+32	55	0	− 55	79	79	0
E. E. 31	Duodenal ulcer 1.75	1+	13	77	122	+45	56	0	− 56	86	85	−1
Mean age, 34	Mean	Mean	10.7	80.9	106.5	+25.6	65.4	3.4	− 62.0	82.4	78.4	−4.0
	S.A.,	S.E.	0.66	0.99	3.49	3.87	13.66	2.67	12.86	3.30	3.99	1.40
Range, 23–47	1.82	S.D.	1.88	2.80	9.89	10.95	38.66	7.56	36.38	9.35	11.29	3.96
Statistical comparison with control series			<0.01	>0.1 <0.2	<0.01	<0.01	>0.5	<0.01	<0.01	>0.5	>0.5	>0.5

TABLE III

Total blood ketone and sugar levels during insulin tolerance tests (0.1 unit insulin per Kg. body weight) in 11 normal medical students with and without cortisone pretreatment

		Total blood ketones *μM acetone/100 ml. blood*						Blood sugar *mg./100 ml. blood*					
		Fasting	Δ ½ hr.	Δ 1 hr.	Δ 2 hrs.	Δ 3 hrs.	Δ 4 hrs.	Fasting	½ hr.	1 hr.	2 hrs.	3 hrs.	4 hrs
Control study	Mean	9.3	−2.4	−2.4	+3.8	+12.9	+16.2	88.0	35.6	67.1	85.4	86.6	85.6
	S.E.	1.38	1.20	1.20	1.72	2.75	3.44	1.66	2.76	3.90	1.90	1.61	1.61
	S.D.	4.47	3.96	3.96	5.50	8.94	11.70	5.51	9.16	12.95	6.30	5.34	5.34
Statistical comparison with fasting levels	p		>0.05 <0.1	>0.05 <0.1	>0.05 <0.1	<0.01	<0.01						
After cortisone	Mean	8.0	−2.4	−0.9	+1.0	+10.7	+12.9	88.7	33.1	70.1	90.4	99.2	99.4
	S.E.	1.38	0.86	1.38	0.86	2.41	3.27	1.46	2.20	4.49	2.44	1.72	1.96
	S.D.	4.30	2.92	4.30	2.75	8.08	11.01	4.84	7.31	14.91	8.12	5.72	6.50
Statistical comparison with fasting level	p		>0.01 <0.02	>0.5	>0.2 <0.3	<0.01	<0.01						
Statistical comparison with control study (p)		>0.5	>0.5	>0.3 <0.4	>0.1 <0.2	>0.5	>0.5	>0.5	>0.4 <0.5	>0.5	>0.1 <0.2	<0.01	<0.01

subjects. After the glucose-octanoate infusion, there was the anticipated elevation of the mean femoral arterial blood glucose level to 143 ± 4.96 (S.E.) mg. per cent and a complete suppression of net splanchnic glucose production. The data on ketone body metabolism in the octanoate period differed from those in the corresponding period in the 1955 series in the following ways: *1*) There was a significant decrease ($p < 0.01$) in the hepatic venous-femoral arterial (HV-FA) blood ketone difference, which was 13.3 ± 2.68 μM per cent compared to 22.2 ± 1.10 μM per cent in the 1955 series; *2*) the mean NSKP was significantly lower ($p < 0.01$) than in the 1955 series, being 106.3 ± 22.4 and 158.0 ± 6.86 μM per minute per M.², respectively; and *3*) the per cent of infused octanoate estimated to be converted to ketone bodies was 9.0 ± 2.02 per cent, a value significantly less ($p < 0.01$) than the 14.9 ± 0.88 per cent found in the 1955 series. In one subject, H.M., the HV–FA blood ketone difference, NSKP and per cent conversion were not significantly different from the 1955 series. In all other subjects the differences were distinct. There were no significant differences in EHBF, SPO_2 or BSP clearance.

Glucose-insulin series (Table II)

Eight subjects were studied before and after the infusion of 20 Gm. of glucose plus 0.1 unit per Kg. body weight HGF-free insulin mixed with sodium octanoate, bringing out only minor differences from the glucose series. Again, the changes were limited to the octanoate infusion period. During the latter, arterial blood glucose levels were lower than those in the glucose series, but NSGP was equally effectively suppressed. Compared to the 1955 series, there were significantly smaller increases in mean arterial and hepatic-venous blood ketone levels and HV–FA blood ketone differences after octanoate infusion in this group ($p < 0.05$, < 0.01 and < 0.05). Similarly, the octanoate infusion induced a smaller net rise in NSKP than in the 1955 series, mean Δ NSKP being + 88.4 ± 6.07 and + 124 ± 7.36 μM per minute per M.², respectively ($p < 0.01$), and the estimated per cent conversion of octanoate to ketone bodies was reduced from 14.9 ± 0.88 per cent to 10.7 ± 0.66 per cent ($p < 0.01$). Although there was no significant difference in the octanoate-period NSKP between this and the glucose series, arterial blood ketone levels were lower in the insulin-glucose group ($p < 0.05$). Indirectly this might suggest that insulin promotes the pe-

ripheral utilization of ketone bodies in the presence of a large carbohydrate load, a concept in distinct contrast to the older observations of Chaikoff and Soskin (3) and Mirsky and Broh-Kahn (4) that neither glucose nor insulin affects the utilization of injected ketone bodies in the eviscerate animal.

While the mean NSKP in this group was significantly lower than that of the 1955 series in the octanoate period, two subjects, P.S. and E.E., attained a net rise in NSKP after octanoate infusion that was comparable to that seen in the 1955 series. In Subject E.E., however, the basal NSKP was approximately three times greater than that in the 1955 subjects. This finding, in the absence of starvation or alkalosis, might reflect the presence of greater anxiety, a potential ketogenic stimulus, than was recognized clinically. In Subject P.S., no explanation is apparent from the data.

Insulin tolerance tests in normal medical students (Table III)

Prior to the hepatic venous catheterization studies reported below in the "Hypoglycemic Series," a pilot study of blood sugar and ketone levels during a standard insulin tolerance test was carried out in 11 normal medical students after an overnight fast.

On the control day, marked hypoglycemia was attained in each subject, with a mean blood sugar level of 35.6 ± 2.76 mg. per cent one-half hour after the insulin. The total peripheral venous blood ketone concentrations showed no significant change from the fasting levels until three and four hours after insulin when there was a net rise of 12.9 ± 2.75 and 16.2 ± 3.44 μM per cent, respectively ($p < 0.01$ for both).[6]

Three days later, the tests were repeated one hour after 200 mg. of cortisone acetate had been administered orally. The degree of hypoglycemia at one-half hour and the rise in venous blood ketone levels during the third and fourth hours were not significantly different from the control day. Blood sugar values three and four hours after insulin (four to five hours after cortisone) rose to significantly higher levels ($p < 0.01$) than did those on the control day. On both the control and cortisone treatment days, there was a tendency for blood ketone concentrations to fall one-half hour after insulin, but this was statistically significant only on the cortisone day ($p < 0.02$). Cortisone pretreatment, in this dosage and timing, did not inhibit the rise in blood ketones during the third and fourth hours after insulin hypoglycemia.

Posthypoglycemic series (Table IV, Figure 1)

Brisk but brief insulin hypoglycemia was induced in six subjects at 7 a.m. by the rapid intravenous injection of 0.1 unit HGF-free insulin per Kg. body weight. Three hours later, at 10 a.m., each was studied in the same manner as the 1955 series before and during the infusion of sodium octanoate. Definite hypoglycemia, approximately 30 minutes in duration, was established in all subjects, the mean lowest blood sugar for the group being 31.0 ± 2.46 (S.E.) mg. per cent.

At the time of study by hepatic venous catheterization, the femoral arterial blood glucose levels and the NSGP had returned to normal control levels, but there were striking changes in ketone metabolism. There were significant increases in the arterial and hepatic venous blood ketone levels and a widening of the HV–FA blood ketone differences, the mean values being 38.8 ± 6.2, 57.6 ± 10.70 and 18.8 ± 5.30 μM per cent, compared to 14.1 ± 1.73, 18.4 ± 2.24 and 4.3 ± 0.62 μM per cent, respectively, in the 1955 series ($p < 0.01$, < 0.01 and < 0.05). Mean control period NSKP was 4.7 times greater than that in the 1955 series of subjects ($p < 0.01$). This is shown graphically in Figure 1. It is interesting to note that mean basal NSKP in the posthypoglycemic subject, 160.7 ± 46 μM per minute per M.^{2}, is almost identical with the mean octanoate period NSKP in the 1955 subjects, 158.0 ± 6.86 μM per minute per M.^{2}. If one could assume that the majority of new ketone bodies produced in the posthypoglycemic state arose from endogenous fatty acids, this might yield a rough estimation of the equivalent quantity of fatty acids being converted to ketones in this state. Despite the elevated basal ketone body levels, this group demonstrated approximately the same increase in blood ketone values

[6] In another group of medical students receiving normal saline I.V., there was a rise in total blood ketones of 5.5 μM per 100 ml. at the end of three hours. This rise was significantly less than that of the hypoglycemic groups ($p < 0.01$).

TABLE IV

Metabolic studies three hours after insulin hypoglycemia

Patient, Age	Diagnosis, Body surface area	Anxiety during test	Total ketones (*μM acetone/100 ml. blood*) Control: Fem. art.	Control: Hep. vein	Control: Δ	During sodium octanoate: Fem. art.	During sodium octanoate: Hep. vein	During sodium octanoate: Δ	Δ Fem. art.	Δ Hep. vein	Δ H.V.–F.A.
yrs.											
W. C. 42	Duodenal ulcer 1.77	2+	46.0	60.0	14.0	87.5	125.8	38.3	+41.5	+65.8	+24.3
L. G. 58	Duodenal ulcer 1.71	2+	32.0	44.8	12.8	69.0	88.5	19.5	+37.0	+43.7	+ 6.7
G. K. 34	Duodenal ulcer 1.91	1+	45.5	71.5	26.0	80.8	112.5	31.7	+35.3	+41.0	+ 5.7
O. R. 30	Acne vulgaris 1.68	2+	51.8	92.8	41.0	76.5	128.5	52.0	+24.7	+35.7	+11.0
A. B. 33	Functional G.I. 1.99	1+	46.0	61.8	15.8	67.8	98.2	30.4	+21.8	+36.4	+14.6
H. H. 33	Asthma 1.49	1+	11.2	14.8	3.6	42.0	71.0	29.0	+30.8	+56.2	+25.4
Mean age, 38	Mean S.A., 1.76	Mean S.E.	38.8	57.6	18.8	70.6	104.1	33.5	+31.8	+46.5	+14.7
		S.D.	6.12	10.70	5.30	6.40	9.1	4.4	3.08	4.9	3.49
Range, 30–58			15.0	26.30	13.0	15.82	22.4	10.9	7.55	12.0	8.49
Statistical comparison with control series		p	<0.01	<0.01	>0.02 <0.05	<0.01	<0.01	<0.01	>0.4 <0.5	>0.5	>0.1 <0.2

Patient, Age	Diagnosis, Body surface area	Anxiety during test	Estimated hepatic blood flow *ml. blood/min./M.²* Control	During sod. oct.	Δ	Net splanchnic ketone production *μM acetone/min./M.²* Control	During sod. oct.	Δ	Net splanchnic oxygen consumption *ml./min./M.* Control	During sod. oct.	Δ
yrs.											
W. C. 42	Duodenal ulcer 1.77	2+	903	866	−37	126	330	+204	30	32	+2
L. G. 58	Duodenal ulcer 1.71	2+	868	790	−78	111	154	+ 43	36	44	+8
G. K. 34	Duodenal ulcer 1.91	1+	914	826	−88	238	261	+ 23	44	37	−7
O. R. 30	Acne vulgaris 1.68	2+	836	825	−11	345	441	+ 96	43	43	0
A. B. 33	Functional G.I. 1.99	1+	749	801	+52	118	230	+112	36	42	+6
H. H. 33	Asthma 1.49	1+	716	644	−72	26	182	+156	27	28	+1

TABLE IV—*Continued*

Patient, Age	Diagnosis, Body surface area	Anxiety during test	Estimated hepatic blood flow *ml. blood/min./M.²* Control	During sod. oct.	Δ	Net splanchnic ketone production *μM acetone/min./M.²* Control	During sod. oct.	Δ	Net splanchnic oxygen consumption *ml./min./M.²* Control	During sod. oct.	Δ
yrs.											
Mean age, 38	Mean S.A., 1.76	Mean S.E. S.D.	831 33.4 81.8	792 31.5 77.1	−39 21.63 53.0	160.7 46.0 112.8	266.3 43.5 106.5	+105.6 27.8 68.0	36 2.70 6.60	37.7 2.65 6.50	+1.70 2.12 5.20
Range, 30–58											
Statistical comparison with control series		p	>0.1 <0.2	>0.1 <0.2	>0.5	<0.01	<0.01	>0.5	>0.5	>0.3 <0.4	>0.5

Patient, Age	Diagnosis, Body surface area	Anxiety during test	Conversion sod. oct. to ketones	Femoral artery glucose *mg./100 ml. blood* Control	During sod. oct.	Δ	Net splanchnic glucose production *mg./min./M.²* Control	During sod. oct.	Δ	BSP clearance % Control	During sod. oct.	Δ
yrs.			%									
W. C. 42	Duodenal ulcer 1.77	2+	24	82	74	−8	61	43	−18	80	81	+1
L. G. 58	Duodenal ulcer 1.71	2+	5	85	80	−5	67	47	−20	97	97	0
G. K. 34	Duodenal ulcer 1.91	1+	3	97	91	−6	64	55	− 9	108	108	0
O. R. 30	Acne vulgaris 1.68	2+	11	94	88	−6	119	122	+ 3	90	89	−1
A. B. 33	Functional G.I. 1.99	1+	15	76	75	−1	73	60	−13	79	75	−5
H. H. 33	Asthma 1.49	1+	15	89	81	−8	50	49	− 1	73	70	−3
Mean age, 38	Mean S.A., 1.76	Mean S.E.	12.2 3.13	87.1 3.17	81.6 2.75	−5.5 1.10	72.3 9.80	62.7 12.1	− 9.6 3.76	87.8 5.34	86.7 5.82	−1.1 0.91
Range, 30–58		S.D.	7.68	7.76	6.74	2.69	24.10	29.7	9.22	13.08	14.25	2.24
Statistical comparison with control series		p	>0.2 <0.3	>0.5	>0.5	0.1	>0.5	>0.5	>0.5	>0.5	>0.4 <0.5	>0.05 <0.1

and NSKP as the 1955 series during the octanoate infusion. In both the control and octanoate periods, mean EHBF, SPO_2 and BSP clearance did not differ significantly from the 1955 subjects.

Cortisone-hypoglycemic series (Table V)

Seven subjects were given 300 mg. cortisone acetate orally at 5 a.m. on the experimental day. Subsequently, they were made hypoglycemic at 7 a.m. and were studied in a manner exactly similar to the "posthypoglycemic series" above. Hypoglycemia was also attained in each of these subjects, the mean lowest blood sugar level for the group being 32.7 ± 2.81 mg. per cent. Due to technical difficulties only a "control period" study was made in Subject D.M.

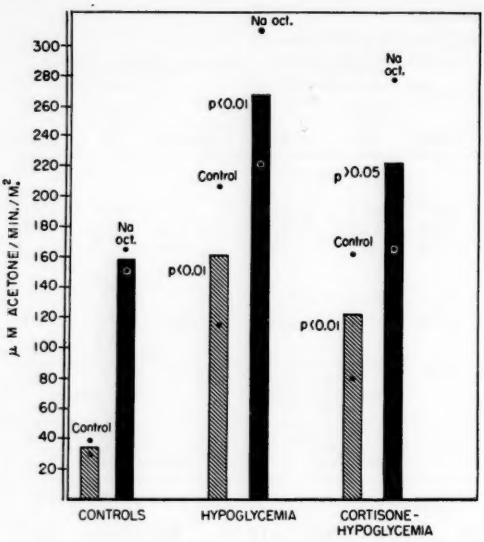

FIG. 1. NET SPLANCHNIC KETONE PRODUCTION BEFORE AND DURING SODIUM OCTANOATE INFUSION IN CONTROL, POSTHYPOGLYCEMIC AND CORTISONE-HYPOGLYCEMIC SERIES OF SUBJECTS (MEAN PLUS OR MINUS STANDARD ERROR)

The p values indicate the statistical comparison between the experimental and control series.

Compared to the posthypoglycemic group, cortisone administered two hours prior to the induction of hypoglycemia did not significantly alter the mean value of any of the measured parameters of carbohydrate or ketone body metabolism. However, closer examination of the ketone data in Table V reveals a remarkable variation of blood ketone levels and NSKP in the individual subjects included in this group. Although comparable degrees of hypoglycemia were attained in each of the seven individuals, Subjects C.P., E.K. and C.F. had the low basal NSKP levels of 37, 41 and 18 μM per minute per M.^{2}, as compared to the mean of 160.7 $\pm$ 46 in the posthypoglycemic group. On the other hand, Subjects E.H. and L.W. varied in the opposite direction with basal NSKP of 299 and 221 μM per minute per M.^{2}. Blood ketone levels revealed a parallel individual variation. Thus, although the mean data in this group indicate that cortisone pretreatment does not affect the ketogenic response to hypoglycemia, the considerable individual variation makes one accept this interpretation with caution.

This group did differ significantly from both the 1955 and posthypoglycemic series in one important respect, the presence of an elevated EHBF in the control period. The mean EHBF in this group was 1,019 $\pm$ 71.92 ml. per minute per M.^{2}, compared with 790 $\pm$ 38 in the 1955 series and 831 $\pm$ 33.4 per minute per M.^{2} in the posthypoglycemic series ($p < 0.01$ and < 0.05). This finding is considered to be a direct or indirect effect of cortisone and is consistent with the previously reported data of Myers and Taylor demonstrating a 43 per cent increase in EHBF following cortisone therapy (5). This effect was partially obscured during the octanoate infusion when there was a fall in EHBF in all three groups.

DISCUSSION

In analyzing the data obtained by this technique it must be kept in mind that we are estimating net *splanchnic* ketone body production. While the viscera and mesentery drained by the portal vein are not the sources of ketone bodies *per se*, the mesenteric adipose tissue unquestionably contributes ketone body precursors as nonesterified fatty acids to the liver. Thus changes in NSKP as herein defined may be determined both by alterations in adipose tissue fatty acid release as well as true hepatic ketogenesis.

The data show that glucose added to an intravenous octanoate infusion significantly reduces the splanchnic ketone production observed when the latter alone is infused in man. The addition of insulin to the glucose-octanoate infusion did not alter the magnitude of this response. This effect of glucose is consistent with the large body of experimental evidence, recently reviewed in detail by Campbell and Best (6), suggesting that carbohydrate exerts its antiketogenic effect by suppressing ketone body production by the liver rather than by influencing peripheral tissue utilization of ketone bodies.

From our data it cannot be determined whether glucose affected the utilization of administered octanoate or simply inhibited endogenous ketone production from fatty acids derived from the liver itself or entering the liver from other sources. Observations by other workers support the conclusion that both mechanisms were influenced to some extent. Dole has clearly shown a sharp decrease

TABLE V

Metabolic studies three hours after insulin hypoglycemia in cortisone pretreated subjects

Patient, Age	Diagnosis, Body surface area	Anxiety during test	Total ketones (μM acetone/100 ml. blood) Control Fem. art.	Control Hep. vein	Control H.V.–F.A.	During sodium octanoate Fem. art.	During sodium octanoate Hep. vein	During sodium octanoate H.V.–F.A.	Δ Fem. art.	Δ Hep. vein	Δ H.V.–F.A.
yrs.											
C. P. 33	Neurosis 1.98	1+	22.2	25.8	3.6	58.5	77.0	18.5	+36.3	+51.2	+14.9
E. K. 29	Neurosis 2.12	1+	12.2	18.2	6.0	23.5	30.8	7.3	+11.3	+12.6	+ 1.3
E. H. 25	Functional G.I. 1.56	1+	67.0	93.0	26.0	122.3	163.3	41.0	+55.3	+70.3	+15.0
W. G. 31	Dermatitis 1.75	1+	49.3	67.0	17.7	94.0	118.2	24.2	+44.7	+51.2	+ 6.5
L. W. 41	Back pain 1.63	1+	52.2	74.0	21.8	93.3	118.3	25.0	+41.1	+44.3	+ 3.2
C. F. 29	Functional G.I. 1.82	2+	12.5	13.2	0.7	38.0	54.8	16.8	+25.5	+41.6	+16.1
D. M. 34	Neurosis 2.00	1+	11.0	18.7	7.7						
Mean age, 32	Mean	Mean	32.3	44.3	12.0	71.6	93.7	22.1	+35.7	+45.2	+ 9.50
	S.A., 1.84	S.E. 8.85	12.58	3.77	15.44	7.73	4.58	6.31	6.26	2.71	
Range, 25–41		S.D. 23.0	37.70	9.8	37.83	18.94	11.22	15.46	15.35	6.63	
Statistical comparison with control series		p	<0.01	<0.01	<0.01	<0.01	<0.01	>0.5	>0.1 <0.2	>0.5	<0.01
Statistical comparison with hypoglycemic series		p	>0.5	>0.4 <0.5	>0.2 <0.3	>0.5	>0.4 <0.5	>0.1 <0.2	>0.5	>0.5	>0.2 <0.3

Patient, Age	Diagnosis, Body surface area	Anxiety during test	Estimated hepatic blood flow ml. blood/min./M.² Control	During sod. oct.	Δ	Net splanchnic ketone production μM acetone/min./M.² Control	During sod. oct.	Δ	Net splanchnic oxygen consumption ml./min./M.² Control	During sod. oct.	Δ
yrs.											
C. P. 33	Neurosis 1.98	1+	1,090	1,164	+ 74	37	206	+169	36	41	+5
E. K. 29	Neurosis 2.12	1+	712	670	− 42	41	49	+ 8	42	33	−9
E. H. 25	Functional G.I. 1.56	1+	1,150	1,098	− 52	299	463	+164	34	43	+9
W. G. 31	Dermatitis 1.75	1+	814	687	−127	144	170	+ 26	30	25	−5
L. W. 41	Back pain 1.63	1+	1,015	1,071	+ 56	221	268	+ 47	32	40	+8
C. F. 29	Functional G.I. 1.82	2+	1,202	1,019	−183	18	170	+152	40	43	+3

TABLE V—*Continued*

Patient, Age	Diagnosis, Body surface area	Anxiety during test	Estimated hepatic blood flow *ml. blood/min./M.²*			Net splanchnic ketone production *μM acetone/min./M.²*			Net splanchnic oxygen consumption *ml./min./M.²*		
			Control	During sod. oct.	Δ	Control	During sod. oct.	Δ	Control	During sod. oct.	Δ
yrs.											
D. M. 34	Neurosis 2.00	1+	1,150			88			40		
Mean age, 32	Mean S.A.,	Mean S.E.	1,019	951	− 46	121.1	221.0	+ 94.3	36.3	37.5	+1.83
	1.84	S.D.	71.92	88.41	40.85	40.77	56.32	30.61	1.75	2.66	2.71
Range, 25–41			187.0	216.6	100.1	106.0	138.0	75.0	4.55	6.51	6.63
Statistical comparison with control series		p	<0.01	<0.01	>0.5	<0.01	>0.05 <0.1	>0.1 <0.2	>0.5	>0.3 <0.4	>0.4 <0.5
Statistical comparison with hypoglycemic series		p	>0.02 <0.05	>0.1 <0.2	>0.5	>0.5	>0.5	>0.5	>0.5	>0.5	>0.5

Patient, Age	Diagnosis, Body surface area	Anxiety during test	Conversion sod. oct. to ketones	Femoral artery glucose *mg./100 ml. blood*			Net splanchnic glucose production *mg./min./M.²*			BSP clearance %		
				Control	During sod. oct.	Δ	Control	During sod. oct.	Δ	Control	During sod. oct.	Δ
yrs.			%									
C. P. 33	Neurosis 1.98	1+	22	102	92	−10	30	76	+46	80	79	−1
E. K. 29	Neurosis 2.12	1+	1	103	96	− 7	154	61	−93	82	75	−7
E. H. 25	Functional G.I. 1.56	1+	17	78	72	− 6	104	52	−52	71	71	0
W. G. 31	Dermatitis 1.75	1+	3	100	87	−13	95	44	−51	60	59	−1
L. W. 41	Back pain 1.63	1+	5	102	96	− 6	66	58	− 8	75	72	−3
C. F. 29	Functional G.I. 1.82	2+	18	97	95	− 2	36	31	− 5	91	86	−5
D. M. 34	Neurosis 2.00	1+		100			121			60		
Mean age, 32	Mean S.A.,	Mean S.E.	11.0	97.4	89.7	− 5.3	86.6	53.6	−27.2	74.1	73.7	−2.8
	1.84	S.D.	3.67	3.38	3.47	2.74	17.38	6.28	19.76	4.45	3.68	1.11
Range, 25–41			9.0	8.79	8.49	6.71	45.2	15.38	48.4	11.57	9.03	2.72
Statistical comparison with control series		p	>0.1 <0.2	<0.01	>0.02 <0.05	>0.2 <0.3	>0.1 <0.2	>0.2 <0.3	>0.1 <0.2	>0.05 <0.1	>0.1 <0.2	>0.5
Statistical comparison with hypoglycemic series		p	>0.5	>0.05 <0.1	>0.1 <0.2	>0.5	>0.5	>0.5	>0.4 <0.5	>0.05 <0.1	>0.05 <0.1	>0.2 <0.3

in plasma nonesterified fatty acids (NEFA) after glucose or insulin administration in man (7). This has been confirmed by Gordon and Cherkes (8). More recent observations by Gordon suggest that glucose and insulin decrease the liberation of NEFA from depot fat, leading to a decline in blood NEFA levels and finally to a cessation of extraction by other tissues (9). It is of particular interest that in three of his subjects studied by hepatic catheterization, positive arterial-hepatic venous blood NEFA differences were abolished by glucose administration. On the other hand, the *in vitro* studies of Weinhouse, Millington and Friedman (10) and Lossow, Brown and Chaikoff (11) have demonstrated that glucose prefeeding significantly reduced the conversion of labeled fatty acid substrates to both CO_2 and acetoacetate by liver slices. Furthermore, Lossow and Chaikoff (12) have observed that carbohydrate feeding significantly lowered the conversion of C^{14} of several injected fatty acids to CO_2 in intact rats. This effect was far more pronounced in the case of longer-chain fatty acids. However, it should be noted that the longer-chain fatty acids were administered in their experiments as unphysiological, complex emulsions in olive oil, whereas the shorter-chain acids such as octanoate were given as the sodium salt. In similar experiments recently reported by McCalla, Gates and Gordon (13) C^{14} labeled sodium palmitate was administered in the form of a serum albumin-bound complex ion which is believed to be the chemical form of endogenously circulating NEFA. Again, there was a marked inhibition of the conversion of intravenously administered palmitate to CO_2 in the carbohydrate fed intact rat, indicating the occurrence of a block in fatty acid oxidation *per se* at the cellular level. Current evidence suggests that endogenous NEFA are composed almost exclusively of these responsive longer-chain fatty acids (14). Since the carbohydrate sparing of shorter-chain fatty acid (octanoate) oxidation is minimal, we believe that the major carbohydrate effect in our experiments was mediated by a suppression of endogenous fatty acid mobilization or catabolism. In this regard it is noteworthy that the NSKP during the glucose-octanoate infusion experiments was roughly equivalent to the NSKP obtained during the administration of octanoate alone minus the endogenous control period NSKP.

Other data reported herein reveal that hypoglycemia is followed in three to four hours by elevated peripheral venous blood ketone levels, confirming the earlier reports of Collip (15) and Somogyi (16). The basis for this ketonemia in man now seems clearly demonstrated in the hepatic catheterization data in the six subjects which showed a 4.7-fold increase in net splanchnic ketone production three hours after hypoglycemia. This observation lends experimental support to the clinical experience that insulin hypoglycemia may initiate ketosis in the diabetic patient. Since the basal levels of splanchnic ketone production were markedly increased in the posthypoglycemic state, it was at first somewhat surprising to find that octanoate infusion in this group simply induced a net rise in ketone production quantitatively similar to that in the control subjects. This suggests that the ability to utilize the exogenous octanoate for ketone body synthesis is neither augmented nor decreased after hypoglycemia, but rather that the high basal level of ketogenesis is a result either of accelerated utilization of hepatic fatty acids or reflects an increased release of fatty acids from adipose tissue and their delivery to the liver where ketogenesis occurs. Support for the latter concept might be obtained by a study of blood nonesterified fatty acid levels after hypoglycemia, but such data are not recorded in the current literature. The basic biochemical events leading to ketosis in the posthypoglycemic state and their endocrine control have recently been reviewed in detail by one of us (17). The present study throws no light on the endocrine mechanisms involved in the ketonemia consequent to insulin hypoglycemia. However, concurrent studies with rats, to be published elsewhere (18), have shown that this ketosis still occurs in the absence of the pituitary, adrenal cortex and adrenal medulla, and hence hormones from none of these glands are essential. Once ketosis has developed it may be promptly abolished by the administration of a second dose of insulin despite continued hypoglycemia. In fasting hypophysectomized-adrenalectomized rats exhibiting marked hypoglycemia (35 mg. per cent) and ketonemia (258 μM per cent), administration of as little as 0.005 unit of insulin promptly restores blood ketone levels to normal without any further fall in the blood sugar. These data suggest that a relative deficiency in in-

sulin secretion during hypoglycemia may be the single most important hormonal factor contributing to the development of ketosis. It is interesting in this regard that tolbutamide, which reputedly stimulates insulin secretion, is also highly effective in depressing ketonemia during fasting and hypoglycemia (19).

Finally our data show that cortisone pretreatment did not significantly inhibit the elevation in basal net splanchnic ketone production in the posthypoglycemic state. We have pointed out that this interpretation must be accepted cautiously in view of the greater individual variations in this group. However, it is consistent with the recent observation of Amatruda and Engel (18) that cortisone pretreatment does not inhibit the development of ketosis following hypoglycemia in the intact rat. In contrast, it has been shown both in man (20) and the rat (21) that cortisone does inhibit fasting ketosis. Two known metabolic actions of cortisone make it a likely antiketogenic agent in the more slowly developing ketosis of fasting. First, cortisone tends to shift the balance of fat metabolism toward a net accumulation of fat either by inhibiting fat breakdown or stimulating fat synthesis. The second is its action in accelerating carbohydrate production by gluconeogenesis in the liver and in regenerating citric acid cycle precursors from protein catabolism. The answer to the discrepancy in the effect of cortisone on ketosis, suppressing fasting but not modifying posthypoglycemic ketosis, probably resides primarily in the nature of the ketogenic stimulus. It seems likely that the metabolic adjustments rapidly brought forth by the intense stimulus of hypoglycemia outweigh the above-mentioned antiketogenic actions of cortisone, particularly in view of the timing of the present experiments.

An alternate explanation is suggested by the recent finding of Scow, Chernick and Guarco (22) that cortisone is ketogenic in the "totally" pancreatectomized hypophysectomized rat and that this ketosis is prevented by insulin. Cortisone thus may be antiketogenic only when it is capable of bringing about an enhanced rate of insulin secretion. In these terms the variable responses in our cortisone treated posthypoglycemic subjects would reflect variations in the capacity of the pancreas in each case to secrete insulin following suppression by exogenous insulin administration.

SUMMARY

1. Further observations on net splanchnic ketone production (NSKP) were made in metabolically normal human subjects before and after the intravenous infusion of sodium octanoate.

2. The addition of glucose or glucose with insulin to the octanoate infusion significantly lowered the net rise in NSKP noted when octanoate alone was infused.

3. Ketosis in the peripheral blood was observed three hours after the induction of insulin hypoglycemia. While at this time basal NSKP was 4.7-fold greater than in the control subjects, the utilization of infused octanoate was unaffected.

4. Cortisone pretreatment failed to suppress the rise in peripheral blood ketone levels and NSKP occurring in the posthypoglycemic state.

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SODIUM DEPLETION IN ADRENALECTOMIZED HUMANS [1]

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Experimental sodium depletion has seldom been studied in man due to the considerable difficulties in inducing loss of sodium. The efficient renal conservation of sodium promoted by aldosterone precludes the development of sodium deficiency via urinary excretion. Salt loss has been achieved by prolonged sweating (1) but this is a relatively vigorous procedure involving complex compensations by the cardiovascular system, kidneys and adrenal glands. Renal salt loss can be easily obtained, however, in adrenalectomized patients receiving maintenance doses of cortisone. Such a subject is relatively normal with respect to a number of metabolic functions (2) but is unable to adjust completely to a low salt diet.

The purpose of this study was to analyze some of the phenomena of sodium depletion as they occur in the adrenalectomized patient maintained with cortisone alone. These were: *1*) the relationship of the initial total exchangeable sodium to the magnitude of the salt loss necessary to induce hyponatremia; *2*) the mechanism of the development of hyponatremia during sodium depletion; and *3*) the question of "inactivation" of intracellular cation.

METHODS

The studies were carried out on six adults on the Metabolic Unit of the James Ewing Hospital. Adrenalectomy had previously been performed on five of the patients for control of metastatic cancer. Two of these patients, T. P. and E. G., had subsequently been subjected to hypophysectomy during relapse. The sixth patient, L. V., had developed classical Addison's disease during the year preceding this study. All patients had normal renal function as measured by blood urea nitrogen (BUN), serum creatinine and 24-hour endogenous creatinine clearance. Only Patient T. P. had edema; this was limited to the arm and was presumably due to recurrent breast cancer involving the chest wall and axilla.

The patients were placed on a low salt diet (6 to 15 mEq. of sodium daily) supplemented with weighed amounts of sodium chloride during the periods of normal salt intake. Water intake was uncontrolled as the analyzed sodium content was insignificant. The studies were performed only during the cool months in order to minimize sweat losses of sodium. The patients were ambulatory but their activity was generally restricted. Each subject was fed from the same lot of food throughout the study and sample diets were analyzed every two weeks. The sodium and potassium content of rejected food was calculated on the basis of these analyses. The dosage of cortisone acetate was constant throughout except in L. V. and is recorded in Table I. No patient received any salt-retaining hormone.

Methods used in this laboratory for the determination of sodium, potassium, chloride, carbon dioxide content, creatinine and BUN have been described (3). Total exchangeable sodium was estimated by the method of Forbes and Perley (4). Urine osmolality was determined cryoscopically with the Fiske osmometer.

The initial body water was assumed to be 60 per cent of the body weight in R. D., a male, who was not sodium depleted. The initial body water in the three women was assumed to be 50 per cent of the body weight. This approximation is derived from an average body water in females of 55 per cent of the body weight and the consideration of sodium depletion prior to this study.

The changes in total base during the period of sodium depletion and at intervals during sodium repletion were calculated by the formula $bB = W_2[B_2] - W_1[B_1]$ (5), where W_1 and W_2 are the initial and final body waters, respectively, $[B_1]$ and $[B_2]$ the initial and final cation concentrations, respectively, and bB the predicted cation balance. The cation concentration is the sum of the serum sodium and potassium concentrations. The serum cation concentration on the first day of the repletion period of M. L. is an interpolated value, as the specimens for that day were lost.

RESULTS

The total exchangeable sodium (Na_e) was measured at the start of each period of sodium depletion. In Figure 1, these values are compared with the amount of salt lost during the experimental period of low sodium intake. For a more valid

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TABLE I

Serum electrolytes and balance data in adrenalectomized patients during sodium depletion and sodium repletion

Patient	Cortisone acetate	Day	Wt.	Sodium			Potassium			Creatinine	Na	K	Cl	CO_2	Creatinine
				Intake	Urine	Feces	Intake	Urine	Feces						
	mg./day		*Kg.*	*mEq./24 hrs.*			*mEq./24 hrs.*			*mg./24 hr.*	*mEq./L.*	*mEq./L.*	*mEq./L.*	*mEq./L.*	*mg. %*
M. L.	50	1	65.5	12.1	103.7	1.62	32.0	62.0	8.5	799	135	6.2	105	27.2	1.2
		2	65.0	9.3	64.6	1.62	32.0	39.5	8.5	798					
		3	64.7	9.3	58.9	1.62	19.01	46.4	8.5	798	133	6.0	103		
		4	64.6	13.2	46.6	1.62	30.0	44.1	8.5	684					
		5	64.1	14.5	40.0	1.62	1.0	46.8	8.5	684	125				
		6	63.7	8.8	21.2	1.62	1.0	39.1	8.5	684	123	5.3	90		
		1	65.0	143.0	68.1	1.33	22.0	53.4	5.03	874					1.7
		2	65.2	138.0	75.2	1.33	37.0	39.1	5.03	874	112	5.6			
		3	64.4	146.0	134.0	1.33	40.0	48.5	5.03	874	121	5.5	94	23.7	1.4
		4	63.2	147.0	99.0	1.33	44.0	43.2	5.03	721					
		5	63.4	147.0	125.8	1.33	46.0	45.7	5.03	721					
		6	63.1	147.0	133.4	1.33	47.0	47.1	5.03	721					
		7	63.3	147.0	118.0	4.90	53.0	46.8	6.10	691	128	5.1	95	26.4	1.1
		8	62.8	148.0	100.0	4.90	53.0	42.4	6.10	691					
		9	63.2	148.0	116.3	4.90	53.0	54.4	6.10	691					
		10	63.6	250.0	175.4	5.60	53.0	59.5	5.60	710	132		102	25.5	1.0
		11	63.4	250.0	159.0	5.60	53.0	51.2	5.60	710					
		12	63.8	250.0	145.2	5.60	53.0	33.7	5.60	710					
		13	64.3	250.0	193.7	5.60	53.0	49.8	5.60	821	131	5.7	102		
		14	64.3	250.0	213.0	5.60	53.0	48.6	5.60	821					
		15	63.9	250.0	194.0	5.60	53.0	56.6	5.60	821					
			64.4								137	5.4	110	25.5	1.0
R. D.	75	1	52.7	12.7	145.0	0.70	103.0	58.5	8.50	887	139	5.6	110	24.0	1.1
		2	51.9	12.7	92.7	0.70	103.0	55.3	8.50	869					
		3	51.6	12.7	154.0	0.70	103.0	60.0	8.50	923	138	5.50			
		4	51.2	10.0	111.0	0.70	83.4	59.8	8.50	853					
		5	51.0	8.0	75.6	0.70	74.2	46.0	8.50	689					
		1		277.0	97.6	0.70	54.3	60.0	8.50	832	120	5.70	96		1.4
		2	51.2	138.0	110.0	0.50	102.0	87.0	7.4	832	122	6.60	91	23.0	
		3	51.3	140.0	125.0	0.50	103.0	88.0	7.4	762	127	5.62	104	25.0	1.2
		4	51.1	140.0	125.0	0.50	103.0	88.0	7.4	762					
		5	50.2	139.0	125.0	0.50	90.0	88.0	7.4	762	134	5.93			
		6	50.9	139.0	128.0	0.50	102.0	69.0	7.4	884					
		7	51.1	135.0	128.0	0.50	90.0	69.0	7.4	884					
			51.2								137	5.42	104		1.0
T. P.	75	1	65.6	14.5	74.9		93.1	54.2		680	138	4.75	100	30.7	1.0
		2	65.6	14.5	78.8		93.1	90.9		680					
		3	64.8	14.5	65.7		93.1	87.4		680					
		4	65.1	14.5	42.7		93.1	57.0		540	133	5.20			
		5	64.8	14.5	46.7		93.1	88.3		540					
		6	64.7	5.6	24.0		42.2	43.9		540					
		7	64.4	2.1	14.7		31.1	38.9		534	130	5.20	100	28.0	1.8
		1	63.6	110.0	46.8		54.0	65.4		584	125	5.95	96	31.0	1.4
		2	64.1	116.0	65.0		93.1	77.8		584					
		3	63.7	116.0	84.0		93.1	78.9		632					
		4	64.8	116.0	94.0		93.1	86.5		632	135	5.60			1.5
		5	64.2	116.0	94.5		93.1	89.1		632					
		6	63.5	116.0	75.7		93.1	86.3		632					
			63.8								138	5.99	103	25.0	1.2
E. G.	75	1	53.6	6.5	149.0		48.2	62.0		529	134	5.61			0.9
		2	53.1	6.5	88.0		48.2	49.4		491					
		3	52.8	6.5	57.0		48.2	44.1		355	130	5.48			1.3
		4	51.5	6.5	57.8		46.5	39.5		368					
		1	50.8	153.0	102.0		37.8	49.3		661	123	6.47			1.3
		2	51.7	119.0	75.1		47.0	51.6		665	130	7.31			
		3	51.0	119.0	65.1		37.0	35.3		421	127	6.33			1.0
		4	51.2	119.0	54.1		47.0	40.8		356					
		5	51.7	119.0	53.3		47.0	45.8		378	130	6.70			1.2
		6	51.9	119.0	80.6		47.0	45.6		389					
		7	51.6	119.0	76.5		47.0	49.1		419					
		8	51.8	119.0	62.8		47.0	40.4		426					
			51.9								136	6.10			1.0

TABLE I—*Continued*

Patient	Cortisone acetate	Day	Wt.	Sodium			Potassium			Creatinine	Na	K	Cl	CO_2	Creatinine
				Intake	Urine	Feces	Intake	Urine	Feces						
	mg./day		*Kg.*	*mEq./24 hrs.*			*mEq./24 hrs.*			*mg./ 24 hr.*	*mEq./ L.*	*mEq./ L.*	*mEq./ L.*	*mEq./ L.*	*mg. %*
L. V.	12.5	1–3	67.5	7.7	101.0	2.1	37.0	29.1	3.6	743	143	4.30	111	24.9	1.0
		4–6	65.5	7.7	52.1	2.1	37.0	34.8	3.6	764	140				
		7–9	64.8	7.7	28.7	2.1	37.0	36.8	3.6	636	137	5.45			
		10–12	63.8	7.7	36.6	2.1	37.0	34.1	3.6	670					1.1
		13–14	63.8	7.7	26.5	2.1	37.0	36.1	3.6	714	137	5.71			
		1–3	63.7	144.0	56.9	3.7	37.0	43.4	6.0	684	134	5.80	109	26.3	1.2
		4–6	53.6	144.0	95.7	3.7	37.0	45.9	6.0	678					
	50	7–9	63.2	144.0	113.0	2.1	37.0	45.6	2.9	780	139	5.32			
		10–12	63.7	144.0	137.0	2.1	37.0	40.6	2.9	874					
M. C.	75	1–3	51.2	9.6	46.3	1.5	58.0	46.2	16.7		141	5.90	106	28.6	1.1
		4–6	50.6	9.6	27.8	1.5	58.0	53.1	16.7	853	139	5.02	105	28.1	1.2
		7–9	50.3	9.6	32.1	1.5	58.0	46.5	15.0	678					
		10–12	49.7	13.0	39.3	1.5	73.0	62.9	15.0	578					
		13–15	49.5	13.0	43.4	2.9	73.0	55.3	14.8	685					
		16–18	49.9	13.0	23.9	2.9	73.0	44.0	14.8	613					
		19–21	48.9	13.0	22.4	2.1	73.0	50.2	12.5	680					
		22–24	48.3	13.0	26.8	2.1	73.0	55.4	12.5	605	136	6.45	102	23.7	1.5
		25–27	48.3	13.0	24.0	2.0	73.0	49.3	18.9	514					
		28–31	48.7	13.0	16.6	2.0	73.0	43.6	18.9	517					
		1–3	47.5	115.0	35.0	1.4	73.0	33.7	12.0	479					
		4–6	48.4	84.2	40.8	1.4	61.1	31.9	12.0	475	132	5.60	96	19.5	1.7
		7–9	47.8	113.0	35.3	1.4	61.7	34.0	12.0	481					
		10–12	48.2	112.0	30.0	3.2	59.8	33.7	21.6	519					
		13–15	48.5	113.0	22.0	3.2	65.3	20.1	21.6	577					
			49.5								146	5.59	106	21.6	1.2

comparison among the patients, these figures have been expressed as a percentage of the predicted normal Na_e using an arbitrary Na_e of 40 mEq. per kilogram of body weight. Thus the light vertically-striped areas in Figure 1 indicate the degree of sodium depletion experienced by these patients before the onset of the period of low salt intake. The dotted cross-hatched area expresses the amount of sodium lost during the balance study as a percentage of the predicted Na_e for each patient.

Patients M. L., T. P. and E. G., who had advancing metastatic cancer, entered the hospital severely depleted of sodium. In spite of these greatly decreased intitial Na_e's, the serum sodium concentrations were within the normal range (Table I). After the loss of relatively little additional sodium (268 mEq., 282 mEq. and 333 mEq., respectively), severe hyponatremia developed (Table II). At this time, six, seven and four days, respectively, the patients had become anoretic, weak and lethargic, and two patients demonstrated muscular cramps and postural hypotension.

In contrast to these subjects, Patients L. V. and M. C. developed symptoms of salt depletion slowly, and in neither case were the symptoms severe nor the hyponatremia profound, although 630 and 614 mEq. of sodium, respectively, were lost (Table II). Their periods of salt depletion were terminated because we did not feel justified in further extending the length of disability. It seemed evident to us, however, that further salt restriction would have been necessary to reach the clinical states experienced by the previous three patients. The initial Na_e was higher in these two latter patients, being normal in M. C. Both patients reduced their urinary losses of sodium gradually but not sufficiently to achieve sodium balance.

The sixth patient, R. D., had a normal Na_e initially, but very rapidly developed severe hyponatremia with the full clinical syndrome. This patient exhibited only minimal renal conservation of sodium and 557 mEq. of sodium was lost in five days (Tables I and II).

As one of the patients, T. P., also had moderately severe diabetes insipidus, it was possible to assess partially the role of antidiuretic hormone in the water retention noted during the development of hyponatremia. In Figure 2 the urine osmolality, urine volume and the 24 hour millios-

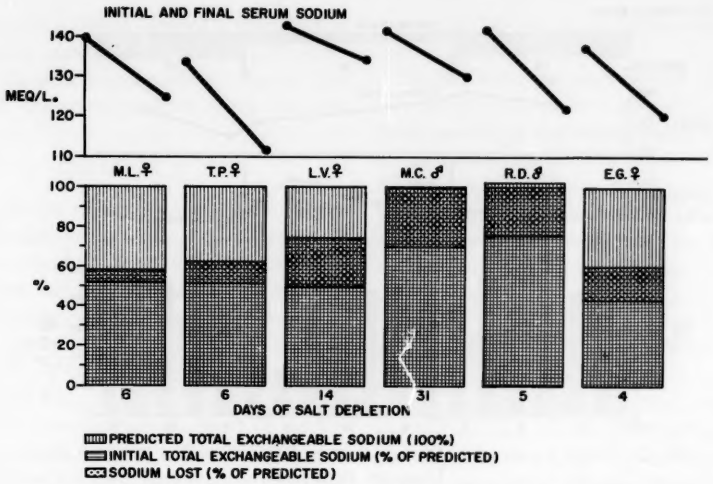

FIG. 1. RELATIONSHIP OF INITIAL Na_e TO SODIUM LOSS AND HYPONATREMIA

mol excretion are plotted throughout the study. The urine volume decreased markedly as a result primarily of poor food intake and correspondingly fewer solutes presented for excretion. Urine osmolality increased slightly, but only to a maximum of 205 milliosmols per liter, a concentration considerably hypotonic to plasma. Therefore, in this patient at least, water retention relative to sodium excretion was noted in the absence of a hypertonic urine and probably at only minimal levels of antidiuretic hormone.

In all patients the glomerular filtration rate (GFR), as estimated by 24 hour endogenous creatinine clearance, fell *pari passu* with sodium depletion and contraction of plasma volume (Table III). This makes interpretation of small changes in urine osmolality of questionable significance, as a rise in urine osmolality may result from a markedly decreased GFR (6).

ΔB_i, the algebraic difference between the predicted cation balance and the observed cation balance, $b_{(Na+K)}$, is recorded in Table III for four

TABLE II

Summary of balances of Na and K in adrenalectomized patients on a low sodium diet and after sodium repletion

Patient	Sodium intake	Days	Initial Na_e	ΔNa	ΔK	Endogenous creatinine clearance at end of period (% initial value)
		no.	*mEq.*	*mEq.*	*mEq.*	
M. L.	Low	6	1,570	− 268	−214	56
	suppl.	15		+ 704	− 89	111
T. P.	Low	7	1,640	− 282	+ 22	42
	suppl.	6		+ 218	− 13	71
E. G	Low	4	1,280	− 333	− 36	48
	suppl.	8		+ 401	− 65	73
R. D.	Low	5	2,270	− 557	+136	55
	suppl.	7		+ 265	+ 42	98
L. V.	Low	14	2,080	− 630	−117	75
	suppl.	12		+ 486	− 9	100
M. C.	Low	31	2,070	− 614	+ 81	40
	suppl.	15		+1,091	+267	69

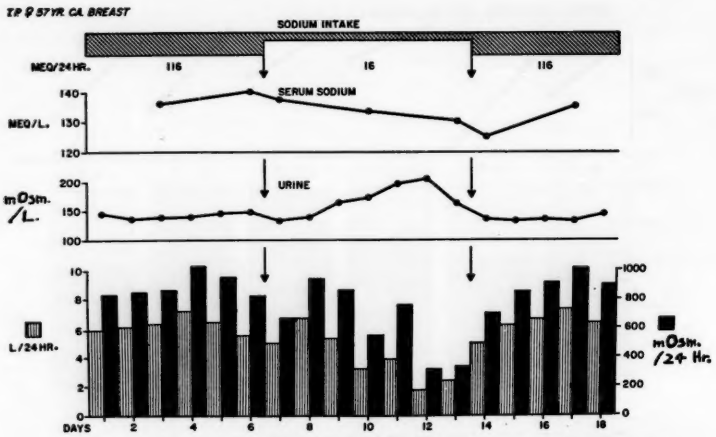

FIG. 2. URINE OSMOLALITY DURING SODIUM DEPLETION IN A PATIENT WITH DIABETES INSIPIDUS

patients. The periods of study were too long in the other two patients for the assumption regarding the equivalence of weight change and alteration in body water to be applicable (*vide infra*). Calculations were made at various intervals during periods of sodium depletion and repletion as well as for the entire period. The predicted cation balance during salt depletion in each case was considerably greater than that measured. This phenomenon occurred in large part during the last interval of the depletion period.

During salt repletion, the converse obtained: Less cation was retained than that predicted. The largest discrepancy in three of the four patients was observed during the first interval, *i.e.*, immediately after increasing the sodium intake.

Similarly, a comparison of the observed serum cation concentration with that calculated for the

TABLE III

Observed vs. predicted cation balances during depletion and repletion of sodium

	Depletion						Repletion					
Patient	Days	Serum cation conc.	Predicted* serum cation conc.	bB	$b_{(Na+K)}$	ΔB_i	Days	Serum cation conc.	Predicted* serum cation conc.	bB	$b_{(Na+K)}$	ΔB_i
		mEq./L.	*mEq./L.*	*mEq.*	*mEq.*	*mEq.*		*mEq./L.*	*mEq./L.*	*mEq.*	*mEq.*	*mEq.*
T. P.	1–3	138	140	−230	−160	− 70	1–3	141	131	+470	+134	+336
	4–7	131	142	−420	−102	−318	4–6	144	148	− 50	+ 71	−121
	1–7	131	144	−650	−262	−388	1–6	144	137	+420	+205	+215
E. G.	1–2	135	135	−170	−259	+ 89	1–4	137	131	+320	+166	+154
	3–4	129	145	−500	−110	−390	5–8	142	143	+ 50	+170	−120
	1–4	129	141	−670	−369	−301	1–8	142	146	+370	+336	+ 34
R. D.	1–2	144	147	−190	−139	− 51	1–4	140	134	+420	+243	+177
	3–5	126	137	−610	−282	−328	5–7	142	142	+ 70	+ 64	+ 6
	1–5	126	139	−800	−421	−379	1–7	142	136	+490	+307	+183
M. L.	1–2	139	138	−180	−206	+ 26	1–6	133	134	+130	+153	− 23
	3–6	122	128	−510	−276	−234	7–9	138	135	+160	+ 91	+ 69
	1–6	122	128	−690	−482	−208	10–15	137	145	+270	+371	−101
							1–15	143	143	+560	+615	− 55

$$*\,[B_2] = \frac{W_1[B_1] + b_{(Na+K)}}{W_2}.$$

end of the depletion period showed a marked discrepancy in each case. During the periods of repletion, this calculation was made at various time intervals and the discrepancies were likewise marked.

DISCUSSION

To our knowledge no previous study has related the total exchangeable sodium and the amount of sodium lost to the development of hyponatremia. It seems evident that in the presence of a depleted total body sodium, small additional losses can rapidly lead to severe hyponatremia. When this occurs about 50 per cent of the total exchangeable sodium has been lost. This situation is the prototype of the spontaneous development of the hyponatremic crisis of Addison's disease. The patient with Addison's disease may endure a gradual depletion of body sodium meanwhile maintaining a normal serum sodium by gradual contraction of plasma volume and extracellular fluid. The imposition of a period of low salt intake or additional salt loss, such as occurs with a gastrointestinal upset, would then quickly precipitate severe hyponatremia.

In addition to the amount of sodium depletion necessary to produce hyponatremia, the rate of salt loss may also influence the degree of hyponatremia and the severity of clinical manifestations. Thus, Patients M. C. and L. V. sustained large losses of sodium, 614 mEq. and 630 mEq., respectively, which occurred gradually and resulted in only modest depressions of the serum sodium concentration. The slow loss of sodium which must have occurred in Patients T. P., M. L. and E. G. prior to the study had not led to hyponatremia.

On the other hand Patient R. D., who had an initial normal Na_e, lost 557 mEq. in five days and the serum sodium level dropped from 139 to 120 mEq. per liter. Nadal, Pedersen and Maddock (7) depleted two normal men of sodium by jejunal drainage causing sodium losses of 365 and 391 mEq. These losses occurred in four and five days, respectively, and although the magnitude of the salt loss was not great, the serum sodium concentrations reached 119 and 117 mEq. per liter. McCance's (1) two studies are intermediate in time so that losses of 980 mEq. and 765 mEq. of sodium in periods of 11 days led to decreases in the serum sodium of 14 mEq. and 13 mEq. per liter.

As McCance has pointed out (1), the loss of salt is accompanied by a loss of water so that the fluid lost during the initial stages of salt depletion is essentially extracellular fluid. With further sodium depletion, volume requirements are somehow "sensed" and water is retained relative to salt, resulting in hyponatremia. It has been suggested that antidiuretic hormone is secreted in response to these volume changes, thus facilitating the relative water retention (8) . The study with the patient with diabetes insipidus is pertinent to this problem. When she developed hyponatremia in response to sodium restriction, urine osmolality reached a value of only 205 milliosmols per liter. Although the hypophysectomized patient does not have a maximum diabetes insipidus (9), there was no evidence of release of antidiuretic hormone. The small increase in urine osmolality could easily have been due to the 60 per cent decrease in GFR (6).

Although any effect of antidiuretic hormone was minimal, a drop in serum sodium occurred. This implies the continued voluntary ingestion of water in the face of a falling serum sodium and presumably decreasing intracellular tonicity as well. Since thirst has generally been related to intracellular hypertonicity, this is a somewhat anomalous response. A sensation akin to thirst has previously been described after salt depletion in man (1) and suggested in the dog (10).

If an increased secretion of antidiuretic hormone was not responsible for the retention of water and development of serum hypotonicity, another mechanism must be sought. The potassium balance was positive during the period of sodium repletion, so that intracellular potassium loss cannot be invoked to explain the hyponatremia as has been suggested by Wynn and Houghton (11). Since the serum CO_2 content did not change, acidosis was probably not a factor. The marked decrease in GFR may be responsible for the water retention in view of studies relating impaired water diuresis to decreases of the GFR in man (12) and the sodium-depleted dog (13).

When salt was returned to these patients the immediate clinical results were dramatic. During the first day, often after retention of less than 70 mEq. of salt, the patients spontaneously vol-

unteered that the cramps had disappeared, dizziness was gone and appetite had returned. McCance (1) also observed similar dramatic effects after restoration of but a small part of the lost salt. These observations suggest that at some critical level of sodium depletion or hyponatremia symptoms become apparent.

The fall in GFR was most marked in those patients who became severely hyponatremic. The GFR in all patients, excluding L. V., averaged 46 per cent of the initial value at the end of the period of salt depletion. This, of course, is not a new finding and, indeed, an elevation of BUN or serum nonprotein nitrogen (NPN) has been seen in all studies of salt depletion. It is worth emphasizing that this finding should be important in the differential diagnosis of the hyponatremias. Thus, a normal serum BUN or NPN in the presence of severe hyponatremia effectively rules out sodium depletion as the cause of the hyponatremia. The converse is not necessarily true, *i.e.*, a patient with poor kidney function may develop dilution hyponatremia. This difference was noted by Wynn (14) in a discussion of hyponatremia. The reports of patients with "asymptomatic" hyponatremia (15, 16) are in accord with this suggestion.

The values obtained for ΔB_i require a discussion of the possible errors involved in these calculations. Assuming values other than 60 per cent for the initial body water will have little effect on bB, the predicted cation balance $(W_2[B_2] - W_1[B_1])$, and therefore little effect on ΔB_i. The most vulnerable assumption is that changes in body weight can be equated with changes in body water. We think this hypothesis is valid because of: *1*) the short periods of study, *2*) a previous control period of six days in three patients during which the weight remained stable, and *3*) the same caloric intake throughout the experiment with the exception of two or three days in three patients. The drop in caloric intake during this brief period of time is reflected in the decreased potassium intake (Table I). In two of the three patients, the drop in caloric intake was small. Patient M. L. had a decrease in caloric intake of 460 and 520 calories on Days Five and Six of the salt-depletion period. The possible weight loss due to this caloric deficit would only be of the order of 0.34 Kg. per day even if half of the calories were supplied by protein. Thus, in the patient with the largest possible weight loss due to poor food intake, the correction would not greatly alter the value for ΔB_i.

During the two days of decreased caloric intake, M. L. had a negative nitrogen balance of 4.2 and 3.1 grams of nitrogen, respectively. This would result in the loss of 21 mEq. of potassium. As this patient had the largest decrease in caloric intake, the possible potassium losses in the other patients due to catabolism of muscle nitrogen were small.

The other possible systematic error was unmeasured losses of sweat sodium. The limited activity and absence of visible sweat make it doubtful that more than 5 mEq. of sodium daily was lost by this route. However, even assuming four times this amount daily does not greatly alter the findings. Furthermore, unmeasured sweat sodium loss would increase ΔB_i during the repletion period. The fact that the large values for ΔB_i occurred during the two to three day interval at the end of the depletion period and at the start of the repletion period, makes the possible sweat losses of lesser significance.

Other errors, such as spontaneous fluctuations of weight and serum sodium, and analytical errors in the determination of sodium and potassium, are random. The uniformly negative values for ΔB_i during depletion and positive values during repletion make it improbable that random errors could account for these effects.

Yannet and Darrow (17) had observed that following acute sodium depletion in cats, the change in the concentration of base in tissue water was two-thirds as great as that predicted on the basis of intra- and extracellular isotonicity. Mellors, Muntwyler and Mautz (18) likewise noted that, on the average, the gain in intracellular water after sodium depletion in dogs was only 60 to 70 per cent of that predicted. Elkinton, Winkler and Danowski (5) found significant discrepancies between the observed and predicted cation balances in a variety of experiments with dogs and humans. In two dogs subjected to sodium depletion, ΔB_i was negative following depletion and positive after repletion. Schwartz, Bennett, Curelop and Bartter (19) similarly observed a large discrepancy between sodium loss and calculated intracellular tonicity. Thus, the findings of our study have ample precedent.

The values calculated for ΔB_i should be of the same magnitude for the periods of depletion and

repletion if the cells had returned to their original state by the end of the study. This was not so in these experiments and the reasons for this are not clear. It is an inadequate explanation to suggest that insufficient time was allowed during the repletion period as the maximum values for ΔB_i were noted during the first interval of this period.

The physical meaning of negative values for ΔB_i has not been clearly defined. It was proposed (5) that this represented the amount of cation rendered osmotically inactive. If an original isoosmotic state and rapid attainment of intra- and extracellular isotonicity is accepted, then this conclusion is warranted. Alternative hypotheses are that during severe salt depletion the cell is able to maintain an internal environment hypertonic to the interstitial fluid or that an initially hypertonic intracellular milieu was increased with salt loss. Our data do not permit us to distinguish among these theories. In any case, it is of interest that this phenomenon may be viewed as a homeostatic mechanism in that maintenance of extracellular fluid volume is permitted at a lower cation concentration with less water entering the intracellular compartment.

Leaf, Chatillon, Wrong and Tuttle (20) and Wynn (21) have shown that large water loads are distributed throughout the total body water and that the predicted cation concentrations closely agree with the observed cation concentrations. In the postoperative period as well, this agreement is good (11). From these data Wynn and Houghton (11) concluded that neither inactivation of cell cation nor departure from intracellular isotonicity occurs. In view of the consistent findings during sodium depletion, these possibilities must still be seriously considered.

SUMMARY AND CONCLUSIONS

Negative sodium balances varying from 241 mEq. to 630 mEq. have been produced in adrenalectomized patients by a low salt diet. As these patients were receiving constant doses of cortisone, the changes noted could be ascribed solely to the effects of sodium depletion. The induction of significant hyponatremia depended upon the extent of depletion of body sodium and possibly on the rate of depletion as well.

The development of hyponatremia was shown to occur in the absence of antidiuretic hormone. The relative water retention which must occur as hyponatremia develops was related to the marked decrease in glomerular filtration rate.

When the observed cation balances were compared with those predicted from the changes in body water and serum cation concentrations, marked discrepancies were noted. These data support the suggestion that under appropriate circumstances either inactivation of intracellular cation or differences between intra- and extracellular tonicity can be demonstrated.

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STUDIES ON THE RELATION BETWEEN SERUM AND SPINAL FLUID BILIRUBIN DURING EARLY INFANCY[1]

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Although bilirubin has been identified in the cerebrospinal fluid of jaundiced patients, the concentration gradient between serum and spinal fluid has not been accurately determined because of the technical difficulties involved in the measurement of minute quantities of bilirubin (1–6). In young infants, the problem is further complicated by having to distinguish between the xanthochromia caused by intracranial hemorrhage from that associated with physiologic hyperbilirubinemia (7). Sharpe and MacLaire (8) and Levinson, Greengard and Lifvendahl (9) noted that 10 to 25 per cent of newborn infants had blood-tinged spinal fluid and assumed that xanthochromia in this age group was the result of hemorrhage. A few years later, Roberts (10) showed by means of the van den Bergh reaction that the pigment was in reality not heme, but bilirubin. Furthermore, he found that the rise and fall of bilirubin during the first few days of life corresponded with the changes in the bilirubin levels in the serum. Using the icteric index, he estimated the concentration of pigment was about 125 times greater in the serum than in the spinal fluid in normal infants, but this ratio is reduced to only 30 times if intracranial hemorrhage should occur. Stempfel and Zetterström (11) have made similar observations on infants with erythroblastosis fetalis. They found that the bilirubin in the spinal fluid in such infants is predominantly indirect-reacting, and showed that a correlation exists between indirect bilirubin and total protein in the spinal fluid.

The blood-spinal fluid barrier for proteins has previously been studied (12–17). Otila (14) found that among premature infants, the spinal fluid protein during the first week of life averaged 100 mg. per cent, and that this did not fall to normal adult levels until about the age of six years. Spiegel-Adolf, Baird, Szekely and Wycis (15) found the mean spinal fluid total protein to be 103.35 ± 37.14 mg. per cent in 14 normal newborns as compared with a value of 28.50 ± 6.06 mg. per cent among 18 older children. More recently, Arnhold and Zetterström (17) undertook paper electrophoresis studies of the proteins in serum and spinal fluid during the neonatal period. They found the relative concentrations to be almost constant and felt that the total concentration of protein directly reflected the functional state of the blood-brain barrier and that all fractions penetrated this barrier at the same rate.

In 1956, Abelson and Boggs (18, 19) described a method for measuring plasma pigments with a peak at 405 to 415 mμ in erythroblastosis fetalis and suggested that the height of this peak may be useful in predicting the severity and prognosis of the disease process. The configuration of these light-absorption curves appear to depend upon the presence of oxyhemoglobins, hematin, and methemalbumin in the serum (20).

The purpose of this present paper is to describe some observations on bilirubin, protein and heme pigments in the spinal fluid of young infants. It is hoped that these may lead to a better understanding of why there is an increased incidence of kernicterus among premature infants and infants with erythroblastosis fetalis.

MATERIALS AND METHODS

A total of 100 newborn infants delivered at the Cook County Hospital were studied. These included: *1)* 34 normal full-term infants weighing five lbs. or more, *2)* 49 normal premature infants weighing four and one-half lbs. or less, and *3)* 17 infants with erythroblastosis fetalis due either to Rh or ABO incompatibility, who were studied prior to exchange transfusion. A small group of older children and adults with jaundice were similarly

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studied and formed a control group. A capillary blood sample was taken in each infant for the determination of serum bilirubin. At the same time, a single lumbar puncture was performed and about 1 to 2 ml. of spinal fluid was withdrawn. The spinal fluid was examined microscopically and failed to show more than five red blood cells per cubic millimeter in any instance.

All of the readings were carried out in a Beckmann DU spectrophotometer. Bilirubin in the serum was determined by a modification of the micro method described by Hsia, Hsia and Gellis (21). The serum was diluted 1:10. In cuvette A (blank), 0.5 ml. of the diluted serum was mixed with 0.1 ml. of diazo blank and 0.6 ml. of methanol. In cuvette B (sample), 0.5 ml. of the diluted serum was mixed with 0.1 ml. of diazo reagent and 0.6 ml. of methanol. Both cuvettes were allowed to stand for 30 minutes and read at 540 mμ. The optical densities (O.D.) were compared with those of a standard curve prepared by dissolving bilirubin (Pfanstiel) in chloroform and treated with diazo reagent in the same manner.

Bilirubin in the spinal fluid was determined by two methods: *a*) Thirty-five hundredths ml. of spinal fluid was placed in a cuvette and read against a chloroform blank at 453 mμ. The O.D. was compared with those of a standard curve prepared by dissolving bilirubin in chloroform and read at the same wave lengths. *b*) The spinal fluid was either diluted 1:2 or used undiluted. In cuvette A (blank), 0.5 ml. of spinal fluid was mixed with 0.1 ml. of diazo blank and 0.6 ml. of methanol. In cuvette B (sample), 0.5 ml. of spinal fluid was mixed with 0.1 ml. of diazo reagent and 0.6 ml. of methanol. These were allowed to stand for 30 minutes and read at 540 mμ. The O.D. was compared with those of a standard bilirubin curve prepared by dissolving bilirubin in chloroform and treated with diazo reagent in the same manner. A close correlation was found between the two methods (r equals +0.96, d.f. equals 94, p less than 0.01).

Since bilirubin during the newborn period is almost always indirect-reacting (22), no attempt was made to measure the "one minute" or "direct" form in the serum. The relation between the concentration of bilirubin in the serum and spinal fluid was expressed in the form of a ratio (SB/SFB).

The heme pigment was analyzed in the following manner: 0.35 ml. of spinal fluid was placed in a cuvette and read at 400, 410, 420, 430, 440, 450 and 460 mμ. The O.D. at 410 mμ was arbitrarily selected to represent the concentration of the heme pigment. No attempt was made to correct for bilirubin.

The total protein in the spinal fluid was determined by

TABLE I

Summary of laboratory findings in newborn infants and older controls

	Premature (4.5 lbs. or less) infants	Full term (5 lbs. or more) infants	Infants with erythroblastosis fetalis	Older controls
Serum bilirubin				
No. studied	49	34	17	17
Mean (*mg.* %)	13.7	6.7	15.9	12.7
S.D.	± 7.7	± 4.0	± 6.4	± 12.8
Range	2.0–44.2	2.1–16.8	4.0–27.4	2.1–55.6
CSF bilirubin				
No. studied	49	34	17	17
Mean (*mg.* %)	0.61	0.24	0.48	0.10
S.D.	± 0.15	± 0.098	± 0.31	± 0.06
Range	0–1.40	0.06–0.49	0.11–1.29	0.015–0.22
SB/CSF ratio				
No. studied	49	34	17	17
Mean	22.7	33.0	44.0	152.5
S.D.	±10.1	±25.5	±35.2	±175.0
Range	6.4–60.0	8.4–104.9	8.0–102.0	28.9–690.0
Total protein				
No. studied	49	34	17	17
Mean (*mg.* %)	167.1	115.3	123.2	61.1
S.D.	±45.0	±33.9	±45.2	± 42.2
Range	81.4–259.2	46.0–194.4	71.3–256.3	15.8–216.0
410 mμ peak				
No. studied	44	31	15	11
Mean (O.D.)	0.422	0.248	0.306	0.115
S.D.	± 0.21	± 0.125	± 0.152	± 0.055
Range	0.086–0.916	0.066–0.518	0.121–0.698	0.020–0.198

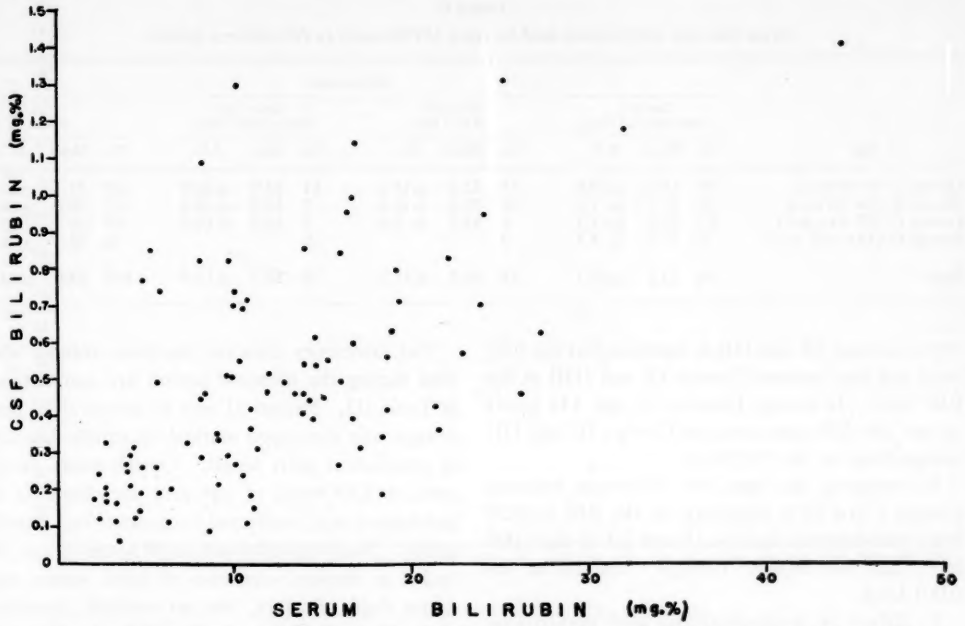

FIG. 1. SERUM BILIRUBIN VERSUS SPINAL FLUID BILIRUBIN (CORRELATION COEFFICIENT [R] = + 0.58)

the method of Waddell (23). The spinal fluid was diluted 1 : 50 and read at 215 and 225 mμ. The total protein was calculated as follows:

$$\text{Total protein } (\mu\text{g./ml.}) = (\text{O.D.}_{215} - \text{O.D.}_{225}) \times 144.$$

RESULTS

The means and standard deviations for each group of infants and of the older controls are summarized in Table I.

I. Relation between serum and spinal fluid bilirubin

A scattergram showing the relationship of spinal fluid bilirubin to serum bilirubin for the 100 young infants is given in Figure 1. The correlation is not close, but significant (r equals + 0.58, d.f. equals 98, p less than 0.01).

It is interesting to note that the SB/SFB ratio does not appear to be significantly influenced by the concentration of bilirubin in the serum. The difference between the mean for the 24 infants with serum bilirubin values of 5 mg. per cent or less and that of the 11 infants with serum bilirubin values of 20 mg. per cent or more is at the 0.05 level.

A. Effect of age. In the present study, it was found that the mean SB/SFB ratio in the controls was 152.5 ± 175.0. This agrees closely with previously published data (4, 5). In contrast, the SB/SFB ratio in the 100 newborn infants was 28.0 ± 24.9. The difference is significant (t equals 2.88, p less than 0.01).

A more detailed analysis of the SB/SFB ratio in the newborn period is tabulated in Table II. In the first two weight groups, the SB/SFB ratio progressively rises as the infant gets older. In the heaviest weight group, the increase can be noted in Groups A and B. Group C consisted of only two cases and the values are not meaningful. The differences between Groups IIA and IIC are significant at the 0.01 level.

B. Effect of birthweight. The SB/SFB ratio was also analyzed in terms of weights at birth in Table II. It can be seen that the SB/SFB ratio rises progressively with an increase in birthweight in each age group. In infants under 48 hours of age, the difference between Groups IA and IIIA is significant at the 0.02 level and that between Groups IIA and IIIA at the 0.05 level. In infants between 49 and 96 hours of age, the difference be-

TABLE II

Serum bilirubin (SB)/spinal fluid bilirubin (SFB) ratios in 100 newborn infants

Age	Birthweight: Group I (less than 4.5 lbs.) No.	Mean	S.D.	Group II (5 to 7 lbs.) No.	Mean	S.D.	Group III (more than 7 lbs.) No.	Mean	S.D.	Sum No.	Mean	S.D.
Group A (0–48 *hrs.*)	10	18.8	±10.6	17	22.6	±16.2	11	43.9	±30.0	38	27.7	±21.4
Group B (49–96 *hrs.*)	20	21.9	± 7.5	10	35.6	±16.8	7	48.8	±25.9	37	30.6	±18.7
Group C (97–144 *hrs.*)	13	26.9	±11.7	4	41.7	± 9.0	2	33.3	±10.9	19	30.7	±12.4
Group D (144–212 *hrs.*)	6	27.3	± 8.5	0			0			6	27.3	± 8.5
Sum	49	22.7	±10.1	31	29.2	±17.5	20	44.5	±15.9	100	28.0	±24.8

tween Groups IB and IIB is significant at the 0.05 level and that between Groups IB and IIIB at the 0.02 level. In infants between 97 and 144 hours of age, the difference between Groups IC and IIC is significant at the 0.02 level.

In summing the data, the difference between Groups I and II is significant at the 0.05 to 0.10 level, that between Groups II and III at the 0.005 level, and that between Groups I and III at the 0.001 level.

C. Effect of erythroblastosis and kernicterus. The means and standard deviations of the SB/SFB ratio in 34 normal full-term infants and 17 full-term infants with erythroblastosis fetalis were compared. Although the SB/SFB ratio tends to be higher in the sensitized infants, the difference is not significant (t equals 1.10, p equals 0.20 to 0.30).

The laboratory data on the three infants who died during the neonatal period are summarized in Table III. Patient 21 was an unsensitized premature who developed marked hyperbilirubinemia in association with sepsis. Opisthotonus developed at 120 hours of age and the diagnosis of kernicterus was confirmed by postmortem examination. Studies performed at 88 hours of age revealed a marked elevation of both serum and spinal fluid bilirubin, but no marked alteration of the SB/SFB ratio. Patient 77 was a full-term infant with erythroblastosis due to anti-E. Clinical signs of kernicterus developed at 130 hours of age and the diagnosis was confirmed by postmortem examination. Studies performed at 121 hours of age showed a high level of bilirubin in the serum, and a slight increase of bilirubin in the

TABLE III

Summary of three patients in present series who died

Patient	Clinical description	Age	Serum bilirubin	CSF bilirubin	SB/SFB ratio	Total protein	410 mμ peak
		hrs.	*mg. %*	*mg. %*		*mg. %*	*O.D.*
			A. Premature infants				
Mean			13.7	0.61	22.7	167.1	0.422
S.D.			±7.7	±0.15	±10.1	±45.0	±0.21
21	Kernicterus (K_I) developed at 120 hrs.; confirmed postmortem	88	32.0	1.17	30.7	233.3	0.136
			B. Infants with erythroblastosis				
Mean			15.9	0.48	44.0	123.2	0.306
S.D.			±6.4	±0.31	±35.2	±45.2	±0.152
77	K_I developed at 130 hrs.; confirmed postmortem	121	27.4	0.63	39.1	133.3	0.350
58	Infant died during 2nd exchange transfusion from K_I. No postmortem performed	29 52	21.4 23.2	0.43 2.69	50.9 8.6	92.9 283.8	0.287 0.195

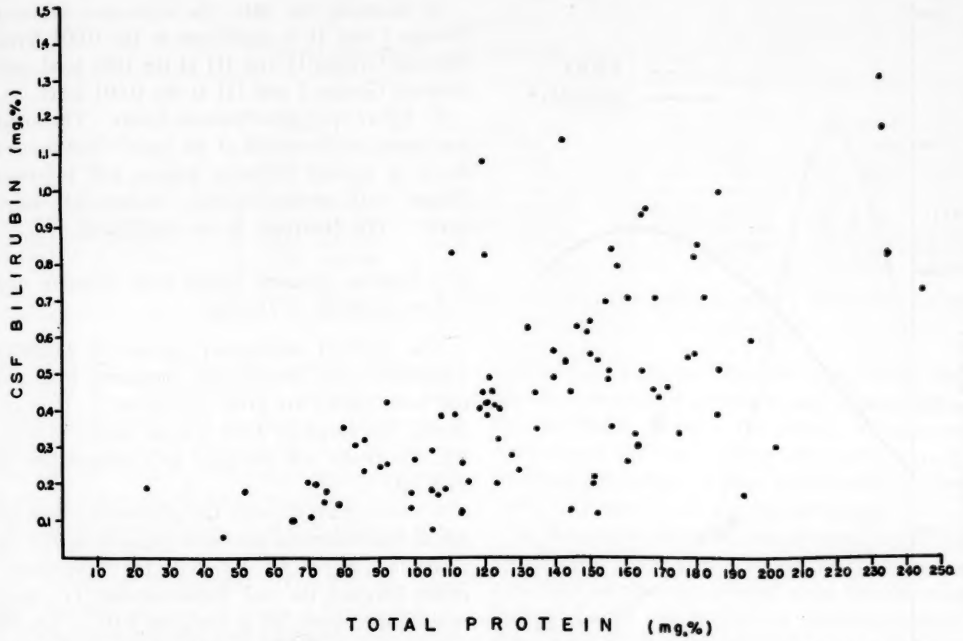

FIG. 2. SPINAL FLUID PROTEIN VERSUS SPINAL FLUID BILIRUBIN (CORRELATION COEFFICIENT [R] = + 0.67)

spinal fluid. Again, there was no great change in the SB/SFB ratio.

The diagnosis on Patient 58 is uncertain. This full-term infant was found to have erythroblastosis against anti-D and was treated by transfusion both at 30 and 53 hours of age. During the course of the second exchange, the patient developed convulsions and expired shortly afterwards. Postmortem permission was not obtained and it is impossible to know whether kernicterus played a role in his death. Laboratory studies showed a marked rise of both the bilirubin and protein in the spinal fluid between the first and second lumbar punctures.

II. Relation between spinal fluid bilirubin and protein

A scattergram showing the relationship of spinal fluid bilirubin to spinal fluid protein for these same infants is given in Figure 2. The correlation is significant (r equals + 0.67, d.f. equals 98, p less than 0.01).

A. Effect of age. The mean spinal fluid protein in the controls subjects was 61.1 ± 42.2. In

TABLE IV

Spinal fluid protein values in 100 newborn infants

Age	Group I (less than 4.5 lbs.)			Group II (5 to 7 lbs.)			Group III (more than 7 lbs.)			Sum		
	No.	Mean	S.D.	No.	Mean	S.D.	No.	Mean	S.D.	No.	Mean	S.D.
		mg.%	*mg.%*		*mg.%*	*mg.%*		*mg.%*	*mg.%*		*mg.%*	*mg.%*
Group A (0–48 *hrs.*)	10	166.4	±47.6	17	134.3	±41.2	11	106.5	±33.9	38	134.7	±46.7
Group B (49–96 *hrs.*)	20	179.8	±41.0	10	108.9	±40.8	7	106.5	±25.8	37	146.8	±24.4
Group C (97–144 *hrs.*)	13	164.2	±44.8	4	129.1	±25.0	2	104.8	±17.8	19	150.6	±44.4
Group D (144–212 *hrs.*)	6	144.8	±24.9	0			0			6	144.8	±24.9
Sum	49	168.6	±16.2	31	125.4	±41.1	20	106.3	±30.1	100	142.7	±48.3

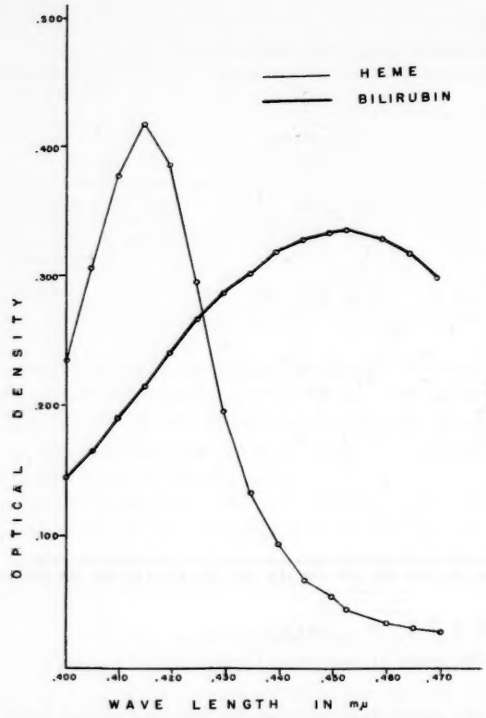

FIG. 3. SPECTRAL ABSORPTION CURVES OF BILIRUBIN AND HEMOGLOBIN

contrast, the spinal fluid protein in the 100 newborn infants was 142.7 mg. per cent. The difference is statistically significant (t equals 6.91, p less than 0.001).

A more detailed analysis of the effect of age upon spinal fluid proteins during the neonatal period is tabulated in Table IV. The differences between the groups are not significant.

B. Effect of birthweight. The spinal fluid proteins were also analyzed in terms of weight at birth in Table IV. It can be seen that the levels progressively decrease depending upon the weight at birth in each age group. In infants under 48 hours of age, the difference between Groups IA and IIIA is significant at the 0.01 level. In infants between 49 and 96 hours of age, the difference between Groups IB and IIB is significant at the 0.01 level and that between Groups I and IIIB at the 0.01 level. In the 97 to 144 hour age group, the difference between IC and IIIC is significant at the 0.01 level.

In summing the data, the difference between Groups I and II is significant at the 0.001 level, between Groups II and III at the 0.05 level, and between Groups I and III at the 0.001 level.

C. Effect of erythroblastosis fetalis. The mean and standard deviations of the spinal fluid protein levels in normal full-term infants and full-term infants with erythroblastosis fetalis were compared. The difference is not significant.

III. Relation between spinal fluid bilirubin and heme pigment at 410 mμ

The spectral absorption curves of bilirubin (Pfanstiel) and hemoglobin (prepared from red cell hemolysate) are given in Figure 3. It is apparent that although there is some degree of overlap, the peaks are separate and independent of each other.

A scattergram showing the relationship between spinal fluid bilirubin and heme pigment at 410 mμ is shown in Figure 4. A high degree of correlation exists between the two measurements (r equals + 0.86, d.f. equals 98, p less than 0.01). On the other hand, no relation was found to exist between spinal fluid bilirubin and the spectral absorption curve of the spinal fluids at 420, 430 and 440 mμ.

A. Effect of age. Although the 410 mμ heme peak was determined in only a few of the control subjects, it would appear that the average values were considerably lower than those seen in newborn infants.

A more detailed analysis of the spinal fluid 410

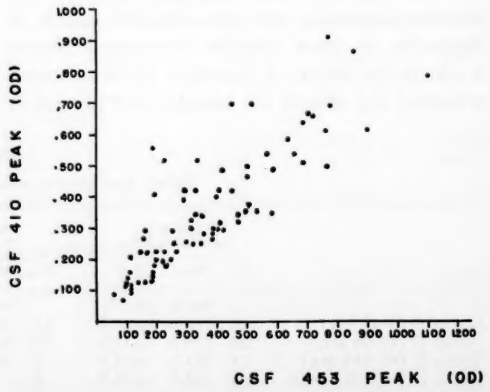

FIG. 4. SPINAL FLUID 410 mμ HEME PEAK VERSUS SPINAL FLUID BILIRUBIN

TABLE V

Spinal fluid 410 mμ heme peak in 90 newborn infants

Age	Birthweight									Sum		
	Group I (less than 4.5 lbs.)			Group II (5 to 7 lbs.)			Group III (more than 7 lbs.)					
	No.	Mean	S.D.	No.	Mean	S.D.	No.	Mean	S.D.	No.	Mean	S.D.
Group A (0–48 *hrs.*)	7	0.297	±0.245	14	0.265	±0.118	9	0.276	±0.141	30	0.276	±0.164
Group B (49–96 *hrs.*)	18	0.472	±0.179	10	0.268	±0.167	7	0.264	±0.158	35	0.372	±0.202
Group C (97–144 *hrs.*)	13	0.462	±0.237	4	0.263	±0.077	2	0.254	±0.000	19	0.402	±0.270
Group D (144–212 *hrs.*)	6	0.298	±0.090							6	0.298	±0.090
Sum	44	0.417	±0.214	28	0.266	±0.134	18	0.269	±0.141	90	0.341	±0.195

mμ heme peak in the newborn period is tabulated in Table V. The differences between the individual groups are not significant. However, it can be seen that the sums of the O.D. at 410 mμ rise with age with the differences between Groups A and B being significant at the 0.05 to 0.02 level, and that between Groups A and C slightly above the 0.05 level.

B. Effect of birthweight. The 410 mμ heme peak was also analyzed in terms of weight at birth. It can be seen that the level falls with increases in birthweight. In infants between 49 and 96 hours of age, the difference between Groups IB and IIB is at the 0.005 level and that between Groups IB and IIIB is close to the 0.01 level. There are too few determinations among infants over 97 hours of age for the analysis to be meaningful. However, in comparing the sums, the differences between Groups I and II and Groups I and III are significant at the 0.005 level.

C. Effect of erythroblastosis fetalis. The means and standard deviations of the 410 mμ peak in 31 normal full-term infants and 15 infants with erythroblastosis fetalis were compared. Although the levels tend to be slightly higher in the sensitized infants, the difference is not significant (t equals 1.26, p 0.20 to 0.30).

DISCUSSION

In the past, reports have appeared showing an alteration in the permeability of the blood-spinal fluid barrier during early infancy to trypan blue (24–26), phosphorus (27, 28), potassium (29) and protein (14, 15, 17). Some workers have attributed these changes to an anatomical disruption of the membrane separating the two compartments due to anoxia and trauma associated with delivery. Others have suggested that the blood-spinal fluid barrier is functional in nature and passage across the membrane requires the action of transport mechanisms such as enzyme systems (30). A transient immaturity of these systems shortly after birth (31) could lead to a similar change.

In the case of bilirubin, the permeability of the blood-spinal fluid barrier during the newborn period must of necessity depend upon the following factors: *1*) the rise and fall of bilirubin levels in the serum, *2*) the concentration gradient of bilirubin on both sides of the membrane at any given time, *3*) the mechanism by which the pigment is transported across the membrane, *4*) the manner by which it is removed from either compartment and *5*) the capacity of the bilirubin to become attached not only to circulatory proteins, but also to surrounding tissues. It is apparent that an arbitrary expression such as the SB/SFB ratio is insufficient to account for each of the variables. However, certain concepts are worthy of speculation in the light of the present study.

The data suggest that the relationship between serum to spinal fluid bilirubin depends less upon the concentration of bilirubin in the serum, and more upon such factors as maturity of the infant at birth (as judged by birthweight), and age of the infant at the time the tests were formed. Among the infants with erythroblastosis, the relationship between the serum and spinal fluid bilirubin remains in the same ratio as among normal infants of comparable maturity. However, the high levels of bilirubin in the serum results in parallel increases in spinal fluid bilirubin, sometimes up to 1 mg. per cent or higher.

No attempt was made to separate bilirubin from its glucuronide in the serum (32). Klatskin and

Bungards (33) have shown that both types of bilirubin remain firmly bound to albumin at levels of pH between 6.0 and 9.0 and separate at levels below 5.0. Since the bilirubin in the serum in this age group is almost always indirect-reacting and all of the infants were clinically free of acidosis, one can assume that bilirubin is firmly bound to protein in the serum and probably enters the spinal fluid as an albumin-bilirubin complex. The positive correlation between spinal fluid bilirubin and total protein would be in agreement with this concept.

The peak at 410 mμ for heme and the peak at 453 mμ for bilirubin appear to be unrelated to each other because: *1*) the spectral absorption curves for bilirubin and hemoglobin are quite distinct as shown in Figure 3, *2*) in the present study, there was no relation between the spectral absorption curve of the spinal fluids at 420, 430 and 440 mμ although a close relationship existed between 410 mμ and 453 mμ, and *3*) there was close agreement on the values for spinal fluid bilirubin measured by two different techniques. The close correlation between the peaks at 410 mμ and 453 mμ in spinal fluid leads to some interesting speculation on a possible biochemical interrelation in the extravascular transport of bilirubin and hematin. It is generally agreed that both hematin and bilirubin form serum protein complexes and are apparently transported in that manner. Since the molecular structure of the two compounds is quite similar, it is not unlikely that they occupy similar sites on the protein molecules with which they tend to combine in complex formation. Tuttle (20) has indicated that in the serum, the protein-hematin complexes are relatively more stable than the corresponding bilirubin complexes. This may explain why there is a relatively poor correlation between the peak for heme and bilirubin in the plasma. On the other hand, in the spinal fluid, we find a rather close correlation between the two measurements. Furthermore, a similar relationship between heme and bilirubin seems to exist in infants with erythroblastosis fetalis and in non-sensitized infants. Since Abelson and Boggs (18) have stressed the height of the 410 mμ peak in the plasma of infants in the former group, one must assume that the blood-spinal fluid barrier must have in some way blocked the passage of most of the heme. It is conceivable that the albumin molecule is "saturated" with both bilirubin and heme groups before passing from the serum into the spinal fluid.

A few years ago, Mollison and Cutbush (34) and Hsia, Allen, Gellis and Diamond (35) reported that kernicterus occurred with greater frequency when the serum bilirubin became markedly elevated in infants with erythroblastosis fetalis. Similarly, the increased incidence of kernicterus among unsensitized prematures (36–42) has been attributed to hyperbilirubinemia on a physiological basis (22, 43). Vogel (44) has suggested that kernicterus occurs in such infants because: *1*) There is an increased permeability of the blood-brain barrier for bilirubin during early infancy, and *2*) the bilirubin is reduced within the brain to mesobilirubin, which gives rise to the cerebral pigmentation in kernicterus. The first of these concepts appears to have been confirmed by the present study. No attempt was made to study the nature of the pigment in the brains of the infants who died. However, in two instances, unusually high spinal fluid bilirubin levels were encountered, and this may have in part been responsible for the development of kernicterus in those infants.

SUMMARY

Simultaneous samples of serum bilirubin and spinal fluid bilirubin, protein and 410 mμ heme peak have been obtained in 100 newborn infants and a number of older controls with jaundice.

A positive correlation coefficient (r equals + 0.58) was obtained between the serum and spinal fluid bilirubin levels. The relation between the serum bilirubin (SB) concentration and spinal fluid bilirubin (SFB) concentration was arbitrarily expressed as the ratio of serum bilirubin/spinal fluid bilirubin. The SB/SFB ratio was the lowest among the smallest and youngest infants. The ratio increased more with weight at birth than with age. The SB/SFB ratio was not significantly altered in infants with erythroblastosis fetalis. Similarly, the concentration of bilirubin in the serum had little effect upon the SB/SFB ratio.

A positive correlation coefficient (r equals + 0.67) was obtained between the spinal fluid bilirubin and total protein. The spinal fluid protein tended to be lower among the more mature infants.

A positive correlation coefficient (r equals + 0.86) was obtained between the spinal fluid bilirubin and the 410 mμ heme pigment. However, no significant difference existed in the 410 mμ heme peak in the spinal fluid of infants with erythroblastosis fetalis and normal full term controls.

The significance of these findings in the pathogenesis of kernicterus was discussed.

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INDUCED TACHYCARDIA: ITS EFFECT UPON THE CORONARY HEMODYNAMICS, MYOCARDIAL METABOLISM AND CARDIAC EFFICIENCY OF THE INTACT DOG[1, 2]

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The relationship of the heart rate to the coronary blood flow had been studied for many years. Thus, in 1926 Anrep and Segall (1) felt that coronary flow was independent of heart rate unless anoxia or excess of carbon dioxide supervened. Katz, Jochim and Bohning in 1938 (2), using an isolated heart preparation, declared that "changes in heart rate produced no change in the coronary inflow and sinus outflow or in the sinus outflow/coronary inflow ratio."

Gregg (3) in 1950 stated that the evidence was conflicting and that no definite statement could be made concerning the effect of rate upon the coronary blood flow. In particular, he himself noted no change when the heart was electrically driven. Wégria, Wang and Glaviano (4) in 1955 stimulated the left atrial appendage and recorded an increase in the coronary blood flow, together with a fall in the systemic arterial blood pressure and cardiac output. In 1956 Laurent, Bolene-Williams, Williams and Katz (5) showed a direct relationship between heart rate and coronary flow, as did Duff, Berglund and Borst (6); a reduction in left coronary flow with slowing of the heart was observed by Starzl, Gaertner and Baker (7).

All of the studies quoted were carried out in isolated heart preparations, heart-lung preparations, or "open-chest" animals. Clearly no ideal circumstance can be encompassed, but it was felt that a study could be designed utilizing an intact animal, anesthetized but breathing room air, in order further to test the inter-relationship of heart rate, coronary blood flow and cardiac efficiency.

METHOD

Nine healthy mongrel dogs, unselected by age or sex, were studied. Their weights varied from 15 to 25 Kg. Morphine sulfate was injected intramuscularly in a dose of 3 mg. per Kg.; one hour later pentobarbital (12 mg. per Kg.) was given intravenously.

By way of the superficial neck veins, cardiac catheters were placed under fluoroscopic guidance in the pulmonary artery, coronary sinus and right atrium. A Cournand needle was placed percutaneously in the femoral artery. The airway was maintained by a cuffed endotracheal tube; apart from the periods of coronary flow study, the animal breathed room air. Each catheter and the arterial needle were connected to a manifold system of stopcocks by short plastic tubes. The volume of these latter was adjusted so that simultaneous samples were obtained from the coronary sinus, femoral artery and pulmonary artery.

Cardiac output was determined by the Fick principle; the expired air was collected for five minutes in a Tissot spirometer connected to the endotracheal tube by a system of one-way flutter valves. The blood samples from the femoral and pulmonary arteries were drawn at the midpoint of the air collection. Femoral and pulmonary arterial pressures were recorded at the beginning and end of the cardiac output. The cardiac rate was determined by standard limb-lead electrocardiograms; respirations were monitored by a pneumograph connected to a strain-gage manometer and recorded on a Sanborn polyviso. Expired air samples were analyzed for oxygen and carbon dioxide in the Scholander apparatus (8). Duplicate checks to within 0.02 per cent were required. Blood oxygen and carbon dioxide contents were measured in duplicate by the method of Van Slyke and Neill (9). The results were required to check within 0.2 volume per cent. Pressures were measured by Statham strain-gage manometers and graphically recorded on a Sanborn polyviso. Mean pressures were obtained by electronic integration. Resistances were calculated from the

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[2] Presented at the National Meeting, American Federation for Clinical Research, Atlantic City, N. J., May, 1957.

[3] John and Mary R. Markle Foundation Scholar.

TABLE I

Metabolic changes consequent to induced tachycardia

	Control*	Tachycardia*	"p" value†
Volume of respiration (*L./min.*)	2.9 ± 0.1	3.3 ± 1.2	>0.6
Oxygen consumption (*cc./min.*)	104 ± 18	116 ± 27	<0.05
Carbon dioxide production (*cc./min.*)	78 ± 17	98 ± 26	>0.1
Respiratory quotient	0.76 ± 0.07	0.77 ± 0.05	>0.7
Arterial O_2 content (*vol. %*)	17.1 ± 2.4	16.9 ± 2.6	>0.7
Mixed venous O_2 content (*vol. %*)	12.9 ± 2.5	12.2 ± 2.7	>0.3
ΔArterial-mixed venous O_2 content (*vol. %*)	4.3 ± 0	4.7 ± 0.6	>0.3
Mixed venous CO_2 content (*vol. %*)	48.5 ± 6.8	47.1 ± 6.7	>0.4
Arterial CO_2 content (*vol. %*)	45.4 ± 7.3	44.0 ± 7.0	>0.4
ΔMixed venous-arterial CO_2 (*vol. %*)	3.0 ± 0.8	3.2 ± 0.7	>0.6

* Mean and standard deviation.
† Tests significance of change.

Poiseuille equation, and works by the laws of dynamics. The formulae used are quoted by Wiggers (10).

Control cardiac output was measured one hour after the intravenous pentobarbital. A steady state was assessed by the presence of sinus arrhythmia, respiratory rate of 12 to 14 per minute, and a steady level of unconsciousness.

Immediately after the control cardiac output the control coronary flow was measured by the nitrous oxide method of Kety and Schmidt (11) as modified for the coronary circulation (12). In all determinations the form of the saturation curve satisfied the criteria of the authors cited. Pressures in the femoral and pulmonary arteries were measured at the mid- and endpoints of the flow. On completion of the control measurements, tachycardia was induced.

Induction of tachycardia: Method. A commercial stimulator [4] was employed. A needle contact was placed subcutaneously over the dog's apex beat. The other terminal was attached to a piano wire stylet traversing the right atrial catheter; the stylet terminated in a metal contact at the tip of the catheter,[5] which was maneuvered against the right atrial wall until stimulation of the latter produced a consistent tachycardia. Stimulation was continued for an average of seven minutes before the cardiac output and coronary flow were re-estimated by the methods already described. The total period of tachycardia averaged 25 minutes. In five dogs tachycardia followed the control study; in four it preceded the control observations.

Left heart weight estimation. The animal was sacrificed at the end of the study and the heart excised. The great vessels and atria were trimmed to their origins, the coronary sinus was ligated, and the coronary arteries to the left heart isolated; into the latter was injected the dye T1824 (Evans' blue). This entered the distribution of the left coronary bed and diffused through the corresponding muscle (13). Using this as a guide, the stained area of the left ventricle and septum (usually about 50 per cent of the latter) was excised and weighed wet. This figure was thought to give a relatively accurate measure of the weight of heart muscle supplied by the left coronary circulation.

The efficiency of the heart (ratio of actual mechanical work of the left ventricle to the energy equivalent of the oxygen consumption of the left heart) was calculated by ordinary thermodynamics applying the formula quoted by Katz and Mendlowitz (14). The oxygen consumption of the arrested heart was excluded in these calculations.

[4] Morris clinical pacemaker, Levinthal Electronic Products, Redwood City, Calif.

[5] This device was designed by Dr. C. A. Castillo.

TABLE II

Systemic and pulmonary hemodynamics consequent to induced tachycardia

	Control*	Tachycardia*	"p" value†
Heart rate	92 ± 19	193 ± 14	<0.001
Cardiac output (*L./min.*)	2.5 ± 0.5	2.5 ± 0.5	>0.9
Stroke volume (*cc.*)	29 ± 8.5	13 ± 3.4	<0.001
Pulmonary arterial pressure (mean *mm. Hg*)	9.0 ± 2.2	11.0 ± 1.8	<0.05
Femoral arterial pressure (mean *mm. Hg*)	95 ± 13	93 ± 9	>0.6
Right ventricular work (*Kg. meter/min.*)	0.3 ± 0.13	0.4 ± 0.14	<0.05
Total pulmonary resistance (*dynes/cm.*$^{-5}$*/sec.*)	286 ± 76	347 ± 66	<0.05
Left ventricular work (*Kg. meter/min.*)	3.3 ± 0.9	3.1 ± 0.7	>0.6
Left ventricular work, stroke (*Kg. meter/min.*)	0.037 ± 0.01	0.017 ± 0.005	<0.001
Total peripheral resistance (*dynes/cm.*$^{-5}$*/sec.*)	3,151 ± 791	3,136 ± 793	>0.9

* Mean and standard deviation.
† Tests significance of change.

RESULTS

In the tables the figures presented are means with standard deviations. A "p" value of 0.05 or less was considered significant. It is implicit that "statistically significant" change is not necessarily "physiologically significant."

Review of all of the factors reported in Table I (General Body Metabolism) shows that only the oxygen consumption rose significantly (104 to 116 cc. per minute); the induction of tachycardia does not apparently change the general metabolism of the animal except in this respect.

In Table II are seen the changes in the systemic and pulmonary hemodynamics due to the induction of tachycardia. The heart rate rises from an average of 92 to 193 beats per minute, and this is not associated with any change in cardiac output although the stroke volume decreases significantly.

The rise in pulmonary arterial pressure, though small, is significant; it is reflected in definite increases both in pulmonary arterial resistance and right ventricular work. The absence of any significant fall in femoral arterial pressure is reflected in the values for left ventricular work and peripheral resistance, neither of which showed any real change. The tachycardia was, however, associated with a definite fall in left ventricular stroke work. It is seen, therefore, that the method of inducing tachycardia did not cause any very profound changes in the general metabolism or systemic hemodynamics.

The results concerning coronary hemodynamics, myocardial metabolism of oxygen and carbon dioxide, and cardiac efficiency are presented in Table III. In reviewing the coronary hemodynamics, it is seen that the coronary blood flow rose significantly and, conversely, the coronary vascular resistance fell. Coronary flow per beat was unchanged. The figures for the oxygen and carbon dioxide metabolism of the heart show that no significant difference in the oxygen or carbon dioxide contents of the coronary sinus blood was achieved. As their arterial counterparts did not change, the differences between the two did not vary either. The cardiac metabolic rates for oxygen and carbon dioxide rose with a high degree of significance, but the cardiac respiratory quotient was unaffected. The oxygen consumption of the weighed heart rose with a high degree of significance. In the face of the tachycardia, however, the cardiac oxygen consumption per beat was unchanged. The measures of efficiency (index of efficiency, coronary blood flow/left ventricular work ratio, or calculated "absolute" efficiency) each showed a significant decrement.

TABLE III

Coronary hemodynamics, myocardial metabolism of O_2 and CO_2, and cardiac efficiency consequent to induced tachycardia

	Control*	Tachycardia*	"p" value†
Coronary flow (*cc./100 Gm./min.*)	81 ± 14	148 ± 34	<0.001
Coronary flow/beat (*cc.*)	0.95 ± 0.26	0.84 ± 0.28	>0.1
Coronary vascular resistance (units)	1.2 ± 0.24	0.7 ± 0.2	<0.01
Coronary sinus O_2 content (*vol. %*)	4.5 ± 1.4	4.2 ± 1.3	>0.3
ΔArterial-coronary sinus O_2 (*vol. %*)	12.3 ± 2.7	13.2 ± 2.9	>0.1
Coronary sinus CO_2 content (*vol. %*)	53.9 ± 7.3	53.4 ± 7.6	>0.6
ΔCoronary sinus-arterial CO_2 (*vol. %*)	9.5 ± 2.6	9.8 ± 2.4	>0.5
Cardiac metabolic rate—oxygen (*cc./100 Gm. myocardium/min.*)	9.4 ± 2.6	18.5 ± 5.5	<0.001
Cardiac metabolic rate—carbon dioxide (*cc./100 Gm. myocardium/min.*)	7.5 ± 1.8	14.1 ± 3.7	<0.001
Cardiac respiratory quotient	0.76 ± 0.08	0.7 ± 0.05	>0.4
Calculated myocardial O_2 consumption, by heart weight (*cc./100 Gm./min.*)	8.3 ± 1.4	17.3 ± 7.4	<0.001
Myocardial O_2 consumption/beat (*cc.*)	0.09 ± 0.037	0.09 ± 0.06	>0.8
Index of efficiency (LV work ÷ $CMRO_2$)	0.38 ± 0.11	0.19 ± 0.06	<0.001
Ratio, coronary blood flow/left ventricular work	25.3 ± 6.7	48.6 ± 11.9	<0.001
Calculated efficiency (% by Joule's Law)	24.0 ± 9.0	12.0 ± 5.0	<0.001

Mean heart weight (left ventricle + free septal wall) = 90.0 Gm.; range, 69–127 Gm.

* Mean and standard deviation.
† Tests significance of change.

The hemoglobin and hematocrit values showed no significant change in the course of the study, nor did the figures for pH [6] in the arterial or coronary sinus blood change significantly. In six dogs estimations of lactate, pyruvate and glucose levels in arterial and coronary sinus blood were made (15–17). Considerable scatter without significant change was found in these values.

DISCUSSION

The variability of results in previous studies concerning the relationship of coronary flow to the heart rate has been commented upon by Gregg (3). This is probably in part due to the "non-intact" preparations used. The present study was designed around an intact animal, attempting to simulate clinical supraventricular tachycardia in which an abnormal pacemaker suddenly becomes dominant. No effort was made to control such conditions as left ventricular work or cardiac output. This perhaps reflects the clinical tachycardias more accurately. In any event, this policy was justified insofar as no significant change accrued in these and associated factors. The increases in body oxygen consumption and carbon dioxide production correspond with similar changes in myocardial oxygen and carbon dioxide metabolism.

It will have been noted that "just significant" increases in pulmonary arterial pressure and right ventricular work are here recorded. There is no *"a priori"* reason to suppose that these changes should affect conclusions concerning the *left-sided* coronary flow. However, utilizing similarly prepared animals and methods, a balloon-tipped catheter was placed in one of the main branches of the pulmonary artery and inflated. Thus a doubling of right ventricular work was obtained, but no significant change in left coronary hemodynamics or myocardial metabolism was found (see results in Appendix). It was felt then that theses to be presented were not vitiated by the pulmonary changes mentioned.

In the acceleration study discussed here, the striking phenomenon is the correlation of the coronary flow with the heart rate. As the arterial/coronary sinus oxygen differences did not vary greatly, the changes in the cardiac metabolic rates for oxygen and carbon dioxide were a function of the increased coronary flow, or primarily of the increase in heart rate.

The tachycardia, in paralleling the coronary flow, did not cause any real change in the myocardial oxygen consumption *per beat* although the effective stroke work fell significantly. Laurent and his colleagues (5) have shown no direct relationship between the stroke work and stroke oxygen consumption. Our study confirms this.

On all counts the cardiac efficiency fell, whether assessed by a calculated "index" value, left ventricular work/coronary flow relationship, or by direct calculation from heart weight. The importance of the heart rate in determining ultimate myocardial efficiency has been emphasized by Sarnoff and co-workers (18), employing a supported isolated heart preparation. Their concept of a tension-time index (aortic systolic mean pressure × duration of systole) suggests that this figure has a direct relationship to the cardiac metabolic rate for oxygen and hence to flow and efficiency. It has been further suggested (19) that the product of rate and systolic pressure, or rate and diastolic pressure, bears a relationship to Sarnoff's tension-time index.

Due to the difficulty of measuring the duration of systole in the arterial tracing as obtained by a

[6] Measured in a Cambridge pH meter.

TABLE IV

Products of rate and systemic pressures

	Control*	Tachycardia*	"p" value†
Rate × systolic pressure	11,593 ± 3,128	21,564 ± 3,565	<0.01
Rate × diastolic pressure	6,728 ± 1,705	13,546 ± 2,178	<0.05
Rate × mean pressure	8,641 ± 2,340	17,299 ± 2,330	<0.05
Cardiac metabolic rate—O_2 (*cc./100 Gm. myocardium/min.*)	9.4 ± 2.6	18.5 ± 5.5	<0.001

* Mean and standard deviation.
† Tests significance of change.

Cournand needle, the tension-time index was not calculable in the present study. However, when systolic or diastolic systemic pressures measured here are multiplied by the heart rate (Table IV), there is a marked increase in these figures in the presence of an induced tachycardia. Since neither the systolic nor diastolic pressures changed greatly, these products are increased primarily because of the increase in heart rate. The figures quoted in Table IV relate directly to the increased coronary blood flow and cardiac metabolic rate for oxygen (r values equal 0.6 to 0.8).

It would seem, therefore, that within limits it is more efficient to deliver the same amount of cardiac work at a slow heart rate than a fast one. In the latter circumstance, energy is inadequately converted to useful work and must appear in some other form, *e.g.*, heat.

SUMMARY AND CONCLUSIONS

In the intact anesthetized dog it appears that the heart rate has a close relationship to coronary flow volume when the heart is electrically accelerated.

Induced tachycardia does not profoundly alter general hemodynamics but it greatly increases coronary blood flow, cardiac oxygen consumption and carbon dioxide production. In the circumstances described, a profound fall in heart efficiency is recorded.

APPENDIX

Effect of increase of right ventricular work upon the left-sided coronary bed (eight studies)

	Control*	Experimental*	"p" value†
Right ventricular work (*Kg./M./min.*)	0.3 ± 0.02	0.6 ± 0.2	<0.01
Cardiac output (*L./min.*)	2.6 ± 1.0	2.7 ± 1.2	>0.2
Coronary blood flow (*cc./100 Gm./min.*)	82 ± 30	81 ± 33	>0.5
Cardiac metabolic rate—O_2 (*cc./100 Gm./min.*)	10.6 ± 5.0	12.5 ± 5.0	>0.2
Cardiac metabolic rate—CO_2 (*cc./100 Gm./min.*)	8.4 ± 5.4	10.6 ± 6.1	>0.1

* Figures are mean and standard deviation.
† Tests significance of change.

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FACTORS INFLUENCING THE CONCENTRATION GRADIENT OF PROTEIN IN CEREBROSPINAL FLUID [1, 2]

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The concentration of protein in the cerebrospinal fluid (CSF) is about 0.5 per cent that of plasma. This difference in concentration is generally considered (1) to be dependent upon the blood-CSF barrier. The normal concentration gradient of protein from a low level in the ventricles, 6 to 15 mg. per cent, to an intermediate level in the cisterna magna, 15 to 25 mg. per cent, to the highest level in the lumbar sac, 20 to 45 mg. per cent (2), has not been satisfactorily explained. To study the nature of this gradient, two groups of experiments were performed, determining: *1*) the exchange of radioiodinated serum albumin (RISA®) between the plasma and the various levels in the CSF, and *2*) the relative concentration of the albumin, globulin and "prealbumin" constituents at these levels, employing zone electrophoretic analysis.

In previous investigations in the dog (3), it has been shown that the plasma albumin exchanges rapidly with the protein in the CSF. Following intravenous injection, RISA® appeared in the cisternal fluid within 20 minutes, reached a maximum at about 20 hours, and subsequently disappeared at a slower rate than in the plasma. In the present study, RISA® was administered intravenously to infants with communicating hydrocephalus to determine if differences in the rates of RISA® appearance exist at different sites along the neuraxis. The demonstration of such rate differences could then be interpreted to indicate nonuniformity of the blood-CSF barrier to protein at these different levels. Similarly, the concentration of albumin in CSF would be expected to be greatest in the region of maximal permeability of the barrier.

METHODS

For the isotopic studies, 12 infants, ranging in age from one to nine months, were studied. In all cases the diagnosis of communicating hydrocephalus was established by ventriculography and dye test or pneumoencephalography. Lugol's solution was administered 24 hours before the study and continued 10 days in order to block thyroidal uptake of the isotopic iodine. Three hundred to 500 microcuries of radioiodinated serum albumin [3] was administered intravenously, four to nine hours prior to combined ventricular, cisternal and lumbar puncture. Eight milliliters of CSF was removed from each of these sites, within a five minute interval. In each case the ventricular fluid was obtained first and the lumbar fluid last. Samples containing more than 100 red blood cells or 10 white blood cells per cubic milliliter were considered contaminated with blood proteins and were discarded. The CSF samples were dialyzed against 0.85 per cent NaCl for 24 hours to remove unbound radioiodine. Radioactivity was measured in duplicate samples with a well-type scintillation counter. The total protein of each sample was determined turbidimetrically using 5 per cent sulfosalicylic acid (4). Specific activity was determined as counts per minute per milligram of total protein, rather than per milligram of albumin, because limitation of sample size precluded separation of the albumin and globulin fractions.

For the electrophoretic analysis of ventricular and lumbar fluids, two groups of patients were studied: infants with communicating hydrocephalus, and children and adults with brain tumors in whom blood-free CSF samples could be obtained. The ages and diagnoses are noted in Table II. Ten to 15 milliliter samples of ventricular and then lumbar fluid were obtained from the patients within a 15 minute period. Samples which were contaminated with blood as defined above were discarded. All samples of cerebrospinal fluid were concentrated by dialysis in cellophane tubing,[4] against a 25 per cent solution of polyvinylpyrollidone.[5] Osmodialysis was carried

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[2] This paper was presented in part at the Annual Meeting of the American Neurological Association, June, 1955, Chicago, Ill.

[3] Abbott Laboratories, North Chicago, Ill.

[4] Visking Corporation, Chicago, Ill.

[5] PVP Type NP K_{30}, General Aniline and Film Corporation, New York, N. Y.

TABLE I

Summary of the data obtained in five infants with communicating hydrocephalus with regard to the total protein content and specific activity at the various levels in the cerebrospinal fluid (CSF), following radioiodinated serum albumin (RISA®) administration

Case	Protein content (*mg. %*)			Specific activity (*cpm/mg.*)		
	Vent. fluid	Cisternal fluid	Lumbar fluid	Vent. fluid	Cisternal fluid	Lumbar fluid
1	11	13	19	107	147	289
2	29	37	45	0	735	914
3	52	55	62	637	829	1,600
4	16		34	78		783
5	105		126	67		301

out at 5.0° C. for 16 hours, resulting in a twenty- to thirtyfold concentration. A final protein concentration in excess of 1 gram per cent is suitable for filter paper electrophoretic analysis. Filter paper electrophoresis was performed under the standard conditions employed for the analysis of serum proteins, viz.: barbital buffer, pH 8.6; ionic concentration, $\mu = 0.075$; voltage gradient, 2.5 V/cm.; duration, 16 hours. Proteins were stained with bromphenol blue dye, and the relative concentrations of globulin, albumin and the prealbumin X fractions were determined by photometric densitometry of the stained electrophoretic patterns.[6] Satisfactory separation of the alpha, beta and gamma subfractions of globulin was not obtained.

RESULTS

In the isotopic studies, of the 12 infants studied, 7 cases were discarded because of the complication of bloody taps, as defined above. The data on

[6] Spinco, Model R, filter paper electrophoresis system, Beckman Instruments, Spinco Division, Palo Alto, Calif.

TABLE II

Summary of the data obtained in nine patients with regard to the electrophoretic analysis of ventricular and lumbar cerebrospinal fluid (CSF)

Patient	Age	Diagnosis	Total protein		X fraction		Albumin		Globulin		X fraction		Albumin		Globulin		A/G	
			V.*	L.†	V.	L.	V.	L.	V.	L.	V.	L.	V.	L.	V.	L.	V.	L.
			mg. %		%		%		%		*mg. %*		*mg. %*		*mg. %*			
1	4 mos.	Communicating hydrocephalus	13	22	3	3	30	45	67	52	0.4	0.7	3.9	9.9	8.7	11.4	0.45	0.86
2	3½ yrs.	Communicating hydrocephalus	36	36	7	3	55	76	38	21	2.5	1.1	19.8	27.4	13.6	7.5	1.44	3.61
3	11 yrs.	Cerebellar astrocytoma	10	21	11	1	46	58	43	41	1.1	0.2	4.6	12.2	4.3	8.6	1.07	1.41
4	8 yrs.	Cerebellar astrocytoma	9	33	3	2	28	65	69	33	0.3	0.7	2.5	21.4	6.2	11.0	0.41	1.97
5	63 yrs.	Cerebellar cyst	16	38	4	1	46	54	50	45	0.6	0.4	7.4	10.5	8.0	17.1	0.92	1.20
6	7 mos.	3rd ventricle tumor	18	65	5	1	26	48	69	51	0.9	0.6	4.7	31.2	12.4	33.2	0.38	0.94
7	9 yrs.	Cranio-pharyngioma	20	56	10	4	45	58	45	38	2.0	2.2	9.0	32.5	9.0	21.3	1.00	1.52
8	61 yrs.	Cerebellar tumor, metastatic	15	92	10	0	42	69	48	31	1.5	0	6.3	63.5	7.2	28.5	0.91	2.22
9	11 yrs.	Medullo-blastoma	16	120	12	3	24	83	64	14	1.9	3.6	3.8	99.6	10.2	16.8	0.37	5.93

* Ventricular.
† Lumbar.

the acceptable 5 cases are summaried in Table I. As seen in this table, in each case the expected increasing concentration of protein from the ventricle to the lumbar sac was observed. This was true in Cases 1 and 4 with normal protein concentrations, as well as in Cases 2, 3 and 5 which displayed abnormally high concentrations.

The specific activity in all five cases was greatest in the lumbar fluid. In the three cases in which cisternal taps were done, the specific activity in the cisternal fluid was intermediate between that of the ventricular and lumbar fluids. In Case 2, despite significant levels of activity in the lumbar fluid, no activity could be detected in the ventricular fluid. In all instances, the specific activity in the ventricular fluid was lower than that measured in fluid obtained from the cistern or lumbar regions.

The total protein values and the relative concentrations of the three major fractions are presented in Table II. Figure 1 shows representative electrophoretic patterns of the ventricular and lumbar fluids obtained in Case 7.

The concentration of the prealbumin X fraction in the ventricular CSF exceeded that in the lumbar fluid in all cases except Case 1, in which the concentrations were equal. The average concentration of fraction X was 7.2 per cent of the total protein in ventricular fluid and 2.0 per cent in lumbar fluid. The amount of fraction X in ventricular and lumbar fluid, however, was variable; in five cases (Nos. 2, 3, 5, 6 and 8) the amount in the ventricular fluid was greater; in four cases (Nos. 1, 4, 7 and 9) the lumbar content was greater. There was no apparent correlation between fraction X content and total protein, albumin, or globulin content. The absence of fraction X from the lumbar fluid of Case 8 is not explained.

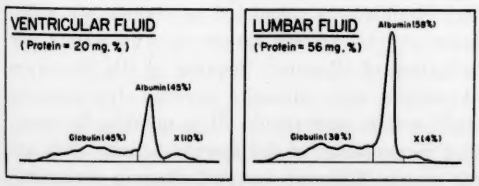

FIG. 1. REPRESENTATIVE ELECTROPHORETIC PATTERNS OF THE VENTRICULAR AND LUMBAR FLUIDS OBTAINED IN CASE 7

The lumbar fluid in all cases contained relatively more albumin than globulin; the ventricular fluids showed just the reverse composition. The average concentration of albumin in lumbar fluid was 62 per cent; of globulin, 36 per cent. The average concentration of albumin in ventricular fluid was 38 per cent; of globulin, 54 per cent. The quantity of albumin was variable but in each case the lumbar content exceeded the ventricular content. The quantity of globulin in lumbar CSF was also increased in eight of the nine cases (exception, Case 2), but the albumin increment was much greater as noted by the A/G ratio which was always greater in the lumbar CSF. The average A/G ratio in ventricular fluid was 0.77; in lumbar fluid, 2.23. In two cases with brain tumor, Cases 8 and 9, and a marked increase in the lumbar total protein, there was marked elevation of the lumbar A/G ratio.

DISCUSSION

The existence of the blood-CSF barrier to protein is clearly evidenced by the marked difference in protein concentration in these fluid compartments. The albumin and globulin of CSF are considered to originate from the plasma except in some disease states, notably neurosyphilis and multiple sclerosis, where the abnormally elevated CSF gamma globulin may be formed within the central nervous system (5). The abnormal plasma globulin, characteristic of multiple myeloma, has also been found in the CSF (5, 6). The gradient in protein concentration from the ventricle to the lumbar sac, in normal and many pathological states, has been well described (2). In a patient with a complete block in the spinal subarachnoid space, Sweet and co-workers (7), employing intravenously injected RISA®, have presented evidence that the increased concentration of protein below the block is secondary to a defect in absorption or removal of protein. The present studies have been devised to explain the protein gradient in cases without obstructive lesions in the spinal subarachnoid space.

The previously demonstrated (3) equilibration of RISA® between plasma and CSF clearly established the dynamic exchange of albumin between these fluid compartments. The present studies have shown that albumin permeates the

blood-CSF barrier at multiple sites along the neuraxis, in agreement with the findings of Sweet and co-workers (7). The choroid plexus does not appear to be essential for the entry of albumin into the CSF. These data supplement the previously reported exchange of sodium (7), chloride (7), potassium (7), glucose (8), urea (9) and water (10) at multiple sites along the neuraxis. It must be recognized, however, as Davson (1) and Bering (10) have emphasized, that the exchange of substances between the plasma and CSF must be distinguished from net changes in volume.

The amount of protein in CSF and the rates of exchange of protein between the blood and the several levels of the CSF are probably dependent upon several independently operating permeability factors. Likewise, the barrier at each of these levels must be considered to be composed of a number of structures, including the capillary endothelium, pia-glial membrane, ependyma, choroid plexus and the arachnoid membranes, each of which may possess different permeability characteristics. The protein concentration gradient from the ventricle to the lumbar sac, in normal and many pathological states, is also dependent upon several different factors including the nature of the barrier at the various levels, mixing within the ventricles and subarachnoid space, and the removal mechanisms that may be operative in the subarachnoid space and at the arachnoid villi. It is difficult to determine the role of each of these factors in the maintenance of the concentration gradient. However, in the present studies, the specific activity was greatest in the lumbar fluid of each patient, the site of greatest protein concentration. We conclude that the concentration gradient of protein must be in part dependent upon the relatively increased permeability of the blood-CSF barrier to albumin in the region of the spinal subarachnoid space. The electrophoretic data support this conclusion in that the albumin content and the A/G ratio were greater in the lumbar CSF than in the ventricular fluid. If a delay in protein removal in the lumbar region was responsible for the higher protein concentration, the globulin concentration would be expected to be greater and the A/G ratio relatively reduced in this region.

In evaluating the appearance of RISA® in the CSF as an indication of differences in permeability along the neuraxis, the surface-volume relationships at the three levels must be considered. In adults the ratio of surface to volume is considered to be greatest in the cisternal region accounting for the more rapid exchange of D_2O in this region than in the ventricular or lumbar region (10). There are no data which quantitatively establish the dimensions of the surface-volume ratios at various levels in the CSF pathways in either normal or pathological states. However, the anatomical relationships of the cisterna magna are such that the surface-volume ratio in this area may be assumed to greatly exceed that ratio in the ventricles or lumbar region. In hydrocephalus, the enlarged ventricular system would tend greatly to depress the rate of entry into the ventricles, but the anatomical relationships in the cisternal and lumbar regions would be relatively unchanged and expected to be such that the surface-volume ratio would remain greater in the cisterna magna than in the spinal subarachnoid space. Thus if permeability to albumin were the same in all regions the specific activity would be greatest in the cisterna magna. The demonstration of the consistently greatest specific activity in the spinal region, the site of greatest protein concentration, strongly suggests that the relatively increased protein concentration in lumbar fluid is in part dependent upon the relatively increased permeability characteristics of the membranes comprising the "barrier" in that region. The nature of these experiments has precluded the study of a normal population. However, the radioisotopic and electrophoretic data suggest this generalization: The occurrence of an increasing concentration gradient of protein along the neuraxis, in the absence of an obstruction in the CSF pathways, is in part dependent upon increased permeability of the membranes adjacent to the site of increased protein concentration.

In the isotope studies the specific activity of each sample was calculated as counts per milligram of total protein rather than as counts per milligram of albumin. Because of the limitation of sample size, albumin globulin fractionation could not be performed. It is notable, however, that recalculation of the specific activity data using several different assumed albumin concentrations, does not modify the conclusion that the tracer most rapidly enters the lumbar CSF. For example, assuming the albumin concentration to

be at least twice as great in the lumbar fluid as in the ventricular fluid does not alter this interpretation.

Those patients with brain tumor and an abnormally elevated lumbar protein had a very marked increase in albumin in the lumbar CSF. There is an increase in the permeability to albumin in the region of brain tumors (11) and this results in an elevated protein throughout the subarachnoid space.

The increasing concentration gradient of protein in the CSF along the neuraxis has, in the past, been attributed to the differential absorption of water in the lumbar subarachnoid space (12). The demonstration by Bering (10) of the very rapid exchange of water between plasma and CSF negates this possibility. The half-times for the exchange of D_2O in seven normal infants and adults were 1.5 to 6 minutes in cisternal fluid, 2 to 37 minutes in the ventricles, and 7 to 38 minutes in the lumbar region.

So-called "stagnation" (2) in the lumbar region has also been set forth as a possible explanation for the relatively increased protein content. The use of a closed system of recording CSF pressure with a strain-gage manometer has revealed the normal arterial pulse pressure in the lumbar CSF to equal 29 mm. saline (13); the ordinary spinal water manometer obscures this finding. A pulse pressure of this magnitude in the lumbar region makes the concept of "stagnation" or poor mixing most unlikely in the absence of a spinal subarachnoid block. The effect of gravity in maintaining the protein concentration gradient has also been suggested as a possible mechanism. The presence of the concentration gradient in infants that are supine is strongly against such a possibility.

Kabat, Moore and Landow (5), in their electrophoretic studies of the CSF proteins, were the first to describe a component with an electrophoretic mobility greater than that of albumin. Subsequent studies by a variety of investigators (14–18), employing several different techniques of concentration of CSF, have confirmed the presence of this fraction. These investigators have variously designated this fraction as fraction X (5, 15, 16), vorfraktion (17), and prealbumin (18). It has been generally considered that this prealbumin fraction is absent from the plasma (14, 15, 19) although Pieper (20) has described the appearance of a comparable constituent in plasma following excessive dilution. Employing immunoelectrophoretic analytical techniques, Gavrilesco, Courcon, Hillion, Uriel, Lewin and Graber (21) and Schultze, Schönenberger and Schwick (22) have also noted the presence of a fast migrating fraction in serum. These workers consider this serum fraction to be identical with the prealbumin fraction of CSF. Uzman and Bering (16) and Schultze and co-workers (22) have attempted to characterize this fraction in CSF chemically and physically. The results of these two studies differ on several points including the amino acid composition, ultracentrifugal sedimentation constants and electrophoretic mobility values. Since these two groups of investigators have employed different isolation techniques, part of the disagreement in their results may be ascribed to these methodological differences.

In the present study, it has been found that the concentration of the prealbumin fraction in the ventricular CSF significantly exceeded the concentration of this component in the lumbar CSF. It should be stressed that this concentration differential is in the opposite direction to the concentration distribution of albumin. Steger (23) and Bauer (24) have noted a similar concentration distribution of this fraction. There are no known correlations between X fraction content in CSF and pathological processes in the nervous system (25).

At present, the site of origin of fraction X in CSF remains obscure. A recent study by Robertson (26) of the soluble proteins of brain, employing an electrophoretic analytical technique, has noted a fraction with a mobility more rapid than that of albumin in brain tissue. Obviously, it is impossible to equate this soluble brain protein with the prealbumin fraction of CSF without more specific physicochemical characterization. The finding of the greater concentration of this fraction in ventricular CSF, however, does suggest the possibility that this fraction may be derived from brain tissue.

SUMMARY

1. Radioiodinated serum albumin was administered intravenously to infants with communicating hydrocephalus. Four to nine hours later, samples of ventricular, cisternal and lumbar cerebrospinal

fluid were obtained within a five minute period. The specific activity was greatest in lumbar fluid, intermediate in cisternal fluid, and least in ventricular fluid.

2. Samples of ventricular and lumbar cerebrospinal fluid were obtained from infants with communicating hydrocephalus and from children and adults with brain tumors. The relative concentrations of the albumin, globulin and prealbumin (X fraction) constituents in these fluids were determined by zone electrophoretic analysis. The lumbar fluid in all cases contained relatively more albumin than globulin as compared with the ventricular fluid. The ventricular fluid contained more prealbumin (X fraction) than the lumbar fluid.

3. These data suggest that the increasing concentration of protein from the ventricle to the lumbar region is a manifestation of greater permeability of the blood-cerebrospinal fluid barrier to proteins in the lumbar region.

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STUDIES ON THE ABSORPTION OF *ESCHERICHIA COLI* ENDOTOXIN FROM THE GASTROINTESTINAL TRACT OF DOGS IN THE PATHOGENESIS OF "IRREVERSIBLE" HEMORRHAGIC SHOCK[1]

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Shock resulting from hemorrhage, which is not reversed by retransfusion, is observed both in man and in experimental animals (1–3). The pathogenesis of this phase of hemorrhagic shock has been the object of extensive investigation (3–11). Endogenous toxic factors liberated in response to prolonged hypoxia, such as VDM (vaso-depressor material), have been implicated (8). More recently, the importance of various bacterial toxins liberated during the course of hemorrhagic shock has been emphasized and it has been postulated that the irreversibility of vascular collapse is principally the consequence of the deleterious action of these substances (6, 7, 9, 11). Several lines of evidence support this thesis.

The oral administration of neomycin or chlortetracycline to dogs before subjecting them to a standardized technique of hemorrhage reduced the mortality from 80 per cent to 20 per cent (7, 10, 12). This effectiveness of neomycin, which is essentially not absorbed from gastrointestinal tract, suggested the importance of the Gram-negative bacterial flora within the lumen of the alimentary tract in the genesis of "irreversibility" (11). The frequent development of extensive necrosis of the mucosa of the small intestine during "irreversible" hemorrhagic shock (1) could enable movement of bacteria or their toxins across an intestinal barrier which is ordinarily impermeable. The observations of Nelson and Noyes of an increased absorption of partially purified *Clostridium botulinum* toxin from the gastrointestinal tract of dogs during "irreversible" hemorrhagic shock lends support to this hypothesis (13). Furthermore, endotoxin liberated by the breakdown of various Gram-negative bacteria, in itself, produces shock. Ebert, Borden, Hall and Gold demonstrated accentuation of the physiological changes associated with endotoxin shock in dogs subjected to the additional stress of minor hemorrhage (14). The converse, extreme increases in the lethality of endotoxin administered after nonlethal hemorrhage, has been observed by Schweinburg and Fine in rabbits (15). Zweifach and Thomas also observed the conversion of a nonlethal episode of hemorrhagic shock in rats into a typical "irreversible" syndrome by the continuous administration of nonlethal doses of endotoxin (16). Such indirect evidence strongly supports the view that bacteria and their products are, in large part, implicated in the pathogenesis of irreversibility during hemorrhagic shock, and more specifically that Gram-negative bacteria through the action of endotoxin are especially concerned in this process.

The studies to be reported here were designed to assess the absorption of radioactive Boivin type endotoxin from various portions of the gastrointestinal tract of dogs subjected to "irreversible" hemorrhagic shock and to evaluate possible increases in the susceptibility of dogs subjected to hemorrhage to small amounts of endotoxin. The development by Braude, Carey, Sutherland and Zalesky (17, 18) of a method for labeling endotoxin with Cr^{51} permits detection in quantities too minute for biological or serological assay (19). Such studies should present direct evidence of the role of bacterial toxins of the type liberated by bacteria normally within the gastrointestinal tract

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in the genesis of "irreversibility" to prolonged hemorrhage.

MATERIALS AND METHODS

A strain of *Escherichia coli* isolated from a patient with fatal bacteremia was used in preparation of endotoxin of the Boivin type according to a previously described technique (19). The isolated endotoxin was either lyophilized or frozen for storage. Six separate lots of endotoxin prepared from this strain of *E. coli* were used.

The radioactive labeling technique generally followed that described by Braude, Carey, Sutherland and Zalesky (17). Precautions were taken to dilute and dialyze the endotoxin in solutions buffered at pH 7.0 to prevent loss of toxicity. Aliquants of endotoxin were diluted in saline solution buffered at pH 7.0 with 0.067 M phosphate buffer. Approximately 1.0 mc. of sterile $Cr^{51}Cl_3$ (Abbott Laboratories, Oak Ridge, Tenn.; specific activity, approximately 1.0 mc. per mg.) for each 300 mouse LD_{50} doses was added to a solution of endotoxin. The endotoxin-$Cr^{51}Cl_3$ mixture was well mixed and incubated at room temperature for 24 hours, then dialyzed with stirring in a bath containing 5 liters of 0.067 M phosphate buffer at room temperature. The bath was changed daily until the dialysate was virtually free of Cr^{51}. This required approximately 48 hours; dialysis was continued 24 to 48 hours longer. The radioactivity of the final endotoxin preparations ranged from 45,300 cpm per mouse LD_{50} to 163,200 cpm per mouse LD_{50}, with the exception of Lot No. 17 in which only 0.3 mc. was added to 300 mouse LD_{50} doses, the resultant final radioactivity being 13,500 cpm per mouse LD_{50}. The radioactivity of these preparations was in the same range (approximately 7,000 counts per second per milligram) as that of the preparations of Braude, Carey, Sutherland and Zalesky labeled with $Cr^{51}Cl_3$ (17).

Studies were performed on each lot of endotoxin after "labeling" with $Cr^{51}Cl_3$ to ascertain that toxicity had not been lost in the labeling procedure. The different lots were standardized on the basis of mouse toxicity and expressed as mouse LD_{50} doses. The LD_{50} of these lots of Cr^{51}-endotoxin varied from 0.08 ml. to 0.3 ml. when given intraperitoneally to 18 to 20 gram albino mice of the Bagg strain. Comparative studies of the toxicity of a representative lot of endotoxin were performed on an aliquant from Lot No. 3. The LD_{50} of the original solution was 0.08 ml.; this volume contained 176 μg. when lyophilized. The nitrogen content of Lot No. 3 was found to be 2.8 per cent by weight as determined by Nesslerization after digestion with selenium-sulfuric acid. This nitrogen content is in the range found by Webster, Sagin, Landy and Johnson for a typical Boivin endotoxin and represents in part glucosamine and amines present in the lipid component (20). The nitrogen content of this preparation is much higher than reported by Braude, Carey, Sutherland and Zalesky, who employed biuret and ninhydrin to test for the presence of proteins (17).

The toxicity of an unlabeled portion of Lot No. 3 when given by systemic intravenous injection was estimated in 12 normal dogs. Two mg. per Kg. killed 10 of 12 animals within 24 hours. The exact LD_{50} was not determined; however, 2.0 mg. per Kg. was chosen as a conservative estimate. The LD_{50} for an average 15 Kg. dog was in the range of 150 mouse LD_{50} doses. These toxicity values are in general agreement with values obtained by other workers, the LD_{50} for 20 gram mice ranging from 0.25 mg. to 1.5 mg., while deaths occurred in dogs following the administration of 5 to 10 mg. per Kg. (21–23).

Radioactivity of various samples was measured in a well-type scintillation counter (Nuclear, Model DS5-5) with an efficiency of approximately three per cent for Cr^{51}. The standard counting interval was 2 minutes in early studies and 10 minutes in later studies. The counting error at the 0.95 level was 23 to 32 cpm for 2 minute counts and 10 cpm for 10 minute counts.

Radioactivity was measured in arterial blood samples (2.0 ml.) drawn at hourly intervals during the shock procedure. At the end of the five hour period of shock, the animals were sacrificed by administration of excess pentobarbital. A midline thoracoabdominal incision was made in the control and shock animals through which a urine specimen was obtained from the bladder using a syringe and needle; portions of the following organs were then removed: adrenal, spleen, liver, paracecal mesenteric lymph node, kidney, muscular portion of diaphragm, lung and trachea. Next, gastrointestinal contents were removed at the following locations: stomach, 24 inches below the pylorus, 24 inches above the ileocecal valve, and descending colon. Approximately 1 gram pieces of the various tissues were weighed to the nearest milligram. The tissues were then digested by adding 2 ml. of concentrated HNO_3 and heating. Foaming was controlled by the addition of a small amount of antifoam (Dow Corning Silicone Defoamer, Antifoam A). The radioactivity of the various samples was measured the day following the shock procedure. The radioactivity for each sample was corrected to counts per minute per gram wet weight.

Experimental procedure

Mongrel dogs of both sexes, averaging 15.0 Kg. body weight (range, 9.1 to 24.5 Kg.) were dewormed, immunized against canine hepatitis and distemper, and kept under standard dietary and environmental conditions at least 21 days prior to the experiments. The dogs were fasted 18 hours before the experiments, which were conducted in an air-conditioned operating room. Control and shock animals were studied in each experiment.

A. Techniques of administration of labeled endotoxin. In the shock studies, labeled endotoxin was administered by two techniques. In the first, a cecostomy or ileostomy was performed; using strict aseptic technique, a midline incision was made, a small polyethylene catheter (outside diameter, 2.4 mm.) was inserted into either the cecum or terminal portion of the ileum employing a Witzel-type repair, and the free end of the catheter was closed and

sutured beneath the skin. At the time of the study, 14 to 21 days after the primary enterostomy, a small skin incision was made and the free end of the catheter located. This technique made it possible to place Cr^{51}-endotoxin into specific areas of the gastrointestinal tract.

In the second technique, dogs were anesthetized lightly with sodium thiopental (19 mg. per Kg.) and under direct laryngoscopy a Levine-type gastric tube was passed into the stomach. Cr^{51}-endotoxin dissolved in 16 to 20 ml. of saline solution followed by 20 ml. of water was gavaged into the stomach. The gavage was performed 2 to 10.5 hours before starting the bleeding, depending upon the portion of the gastrointestinal tract in which the greatest Cr^{51}-endotoxin concentration was desired. By 10.5 hours the maximum radioactivity was present in the large intestine.

The dogs received from 53 to 133 mouse LD_{50} doses with an average of 96 mouse LD_{50} doses through either stomach or enterostomy tube.

Of the two methods used for the administration of endotoxin, gavage through a stomach tube proved more satisfactory than injection through either an ileostomy or cecostomy. It was difficult to be certain in the enterostomy technique that a portion of the labeled endotoxin had not leaked into the peritoneal cavity from which systemic absorption would occur. The only problem encountered in the interpretation of results following gavage of labeled endotoxin was aspiration during the five hour period of study. This was controlled by using endotracheal intubation in all dogs and by determining the presence of radioactivity in the trachea and lungs. Dogs were excluded from tabulation which were found to have higher levels of radioactivity in the tracheal secretions than in lung.

B. Technique and features of shock procedure. At varying intervals after gavage or immediately after injecting endotoxin into the enterostomies, control and shock animals were lightly anesthetized with sodium thiopental (19 mg. per Kg.) and an endotracheal tube inserted. Then, using strict aseptic surgical technique, a sterile polyethylene cannula (1.8 mm. inside diameter) was inserted via the right femoral artery into the aorta. A two side-arm manifold (consisting of two three-way stop cocks) was connected to the cannula. A U-type mercury manometer was connected to one side-arm by means of sterile plastic tubing. A sterile disposable plastic donor set and sterile pyrogen-free 1,000 ml. glass reservoir (a standard 1,000 ml. saline solution bottle emptied just prior to use) was connected to the second side-arm. The remaining connection on the manifold was used to obtain blood samples. The dead space in the entire system was filled with sterile saline solution containing 0.4 mg. per ml. of sodium heparin. Each dog received 2.5 mg. per Kg. of sodium heparin intra-arterially, and 40 mg. of sodium heparin was injected into the reservoir. Control and shock dogs were subjected to the same operative procedure and received the same medications except that a reservoir was not connected to the cannula in the control animals.

After complete recovery from the short period of anesthesia, the dog to be "shocked" was allowed to bleed spontaneously into the sterile reservoir system until the mean arterial blood pressure was 40 mm. Hg. Under constant observation, the blood pressure was maintained at 40 mm. Hg using manual reinfusion under pressure when necessary. The period of shock was five hours, unless the animal died during this interval. No additional anesthesia was required in the shock dogs. Control animals usually required an additional 100 mg. of sodium thiopental at one and three hours after the start of the experiment. The significant features of the shock procedure are tabulated in Table 1. The mean values for the per cent of the maximum bled volume which had to be reinfused during the five hour period of hemorrhage to maintain the mean arterial blood pressure at 40 mm. Hg, the per cent "uptake," correspond quite clearly to the values observed by Fine, Frank, Schweinburg, Jacob and Gordon (7).

Hemorrhagic shock which is associated with a 40 per cent "uptake" of the maximum volume of shed blood is generally accepted as being "irreversible," even if at that time the entire volume of shed blood is reinfused. Hemorrhage into the lumen of the gastrointestinal tract, usually most marked in the duodenum but often

TABLE I

Observations on the shock procedure in dogs *

Route of toxin administration	No. animals	Maximum bled volume	Uptake†	Deaths during procedure
		ml./Kg.	%	
Gastric tube	16	53.6	38	3
Cecostomy	9	53.5	53	1
Ileostomy	1	47.5	35	0
Mean for all groups‡	26	53.3 ± 2.4	43 ± 6	4

* Dogs were allowed to bleed rapidly into a reservoir until the mean arterial blood pressure reached 40 mm. Hg. This blood pressure was maintained for a five hour period with further bleeding or reinfusion as required. Includes five dogs in which intraperitoneal leakage of labeled endotoxin occurred during injection through cecostomy tube.

† Per cent of maximum bled volume reinfused during the five hour experimental period to maintain mean arterial pressure at 40 mm. Hg.

‡ Mean plus or minus standard error of the mean.

TABLE II

Absorption and distribution of radioactivity from the gastrointestinal tract of normal dogs following the administration of free chromic^{51} chloride

No. animals	Radioactivity in tissue (mean *cpm/Gm.* ± *S.E.M.*)						
	Blood*	Urine	Liver	Spleen	Kidney	Adrenal	Lung
7	77 ± 29.4	6,623 ± 3,100	57 ± 27.9	28 ± 18.5	56 ± 29.4	37 ± 18.1	52 ± 11.9

* Five hour blood sample; earlier values were similar.

found extending to the ileocecal region, was observed in the majority of the shocked animals

C. Determination of the diffusibility of free Cr^{51} ions across the gastrointestinal mucosa and systemic distribution in normal dogs. Sterile chromic^{51} chloride diluted with saline was administered by gavage to seven lightly anesthetized dogs to determine whether free Cr^{51} crossed the gastrointestinal mucosa of the normal dog and, if so, the distribution of radioactivity in various body tissues. The volume and total radioactivity administered was similar to that used with labeled endotoxin (20.0 ml. containing approximately 10,000,000 cpm). The technique of administration, duration of study, and tissue analyses were the same as those used in the shock preparation. Significant radioactivity was detected in the tissues of each dog (Table II). The radioactivity in the blood probably accounts for the radioactivity detected in the other tissues. Very high levels of radioactivity were noted in the urines of these animals (6,623 ± 3,100 cpm per ml.). In two animals radioactivity was not detected in the gastrointestinal tract at the segment 24 inches below the pylorus; in the remainder, radioactivity was detected at this level or within the ileum. The radioactivity in the urine of the former animals was 470 cpm per ml., while in the latter, the radioactivity averaged 9,085 cpm per ml. of urine, suggesting that absorption was greater below the duodenum.

The distribution pattern of radioactivity throughout the body noted in two dogs given chromic^{51} chloride into a foreleg vein was similar to that observed following absorption from the gastrointestinal tract.

D. Distribution of labeled endotoxin in shocked and normal dogs following splanchnic or systemic administration. The distribution of radioactivity throughout the body following the systemic or portal intravenous or intraperitoneal injection of Cr^{51}-labeled endotoxin into dogs during shock was compared with the distribution following systemic intravenous injection or the absorption of free chromic^{51} chloride from the gastrointestinal tract. The radioactivity following the parenteral administration of Cr^{51}-labeled endotoxin was highest in the liver, spleen and lungs, while the radioactivity in the urine was minimal in contrast to the distribution noted with free chromic^{51} chloride (Table III). The distribution of radioactivity in normal dogs after the systemic or portal intravenous administration of Cr^{51}-endotoxin was the same as during shock and is essentially the same as noted in rabbits (16). The differences in the renal excretion of Cr^{51}-endotoxin and free $Cr^{51}Cl_3$ suggest that high levels of radioactivity in the urine of animals receiving Cr^{51}-

TABLE III

*Distribution of radioactivity following the parenteral administration of Cr^{51}-labeled endotoxin to dogs during shock**

Dog no.	Route of injection	Radioactivity in tissue (*cpm/Gm.*)						
		Blood	Urine	Liver	Spleen	Kidney	Adrenal	Lung
2371	Intravenous	16	46	1,430	760	32		160
2125	Intraperitoneal	24	0	1,575	150	0	75	22

* Representative animals, which were maintained at a blood pressure of 40 mm. Hg for five hours. Cr^{51}-endotoxin was administered two hours after the onset of shock. The quantity of radioactivity (endotoxin) administered was not the same for each animal, but was approximately 0.10 of the estimated LD_{50} dose for the dog.

TABLE IV

Relationship between radioactivity of liver tissue and quantity of Cr^{51}-labeled endotoxin administered by portal vein injection

Animal no.	1	2	3	4	5	6
Total radioactivity injected (*cpm*)	399,000	199,500	149,620	99,750	74,810	37,410
Radioactivity in liver* (*cpm/Gm.*)	540	356	290	224	112	48

* Average radioactivity determined in duplicate samples from both the right and left lobes of the liver.

TABLE V

Radioactivity of $Cr^{51}Cl_3$-endotoxin preparations

Endotoxin lot no.	Radioactivity* (*cpm/mouse LD_{50}*)	No. shock dogs given each lot	Minimum detectable quantity of endotoxin† (Fraction of LD_{50}) Mean	Range
3	74,270	3	0.0018	0.0011–0.0030
8	64,200	2	0.0021	0.0013–0.0035
14	163,200	5	0.0008	0.0005–0.0014
15	96,750	5	0.0014	0.0008–0.0023
17	13,500	4	0.0108	0.006 –0.016
31	45,360	2	0.0014	0.0008–0.0023

* Counts per minute per volume of $Cr^{51}Cl_3$ endotoxin which was determined to be LD_{50} for 18 to 20 Gm. mice of Bagg strain.

† Minimum detectable quantity of endotoxin was based on the assumptions that liver weight was 2 per cent of body weight, that 47 per cent of radioactivity localized in the liver following portal vein injection, and that the LD_{50} for the dog was 150 mouse LD_{50} doses. The radioactivity necessary to exceed the 0.95 counting error of 32 cpm or 10 cpm depending on counting interval was calculated. The mean was based on 15 Kg., the range 9.1 Kg. to 24.5 Kg., which were the weights of the dogs in these studies.

labeled endotoxin is an indication that the Cr^{51} label had been split from the endotoxin.

E. Determination of lower limit of detectability of labeled endotoxin following injection into portal vein. The minimal quantity of Cr^{51}-labeled endotoxin which could be detected in body tissue was ascertained by the injection of varying amounts under direct vision into the portal veins of six lightly anesthetized (sodium thiopental, 19 mg. per Kg.) normal dogs. The animals were sacrificed after a period of 30 minutes and the radioactivity present in the liver, spleen, adrenals, kidneys, lungs, blood and urine was determined. The variability in cpm per gram wet weight between various lobes of the liver was within the counting error of 32 cpm. Assuming the liver weight to be two per cent of the total body weight, 47.3 ± 2.4 per cent of the total injected radioactivity was present in the liver. The radioactivity present in the other tissues was variable and was not well correlated with the quantity of Cr^{51}-labeled endotoxin injected. A linear regression was calculated for the relationship between radioactivity per gram of liver (x) and the total radioactivity injected into the portal vein (y), $y = 705x - 24{,}500$ with the correlation coefficient $r = +0.9617$ (Table IV). This linear regression enables calculation of radioactivity absorbed into the portal vein based upon the radioactivity per gram of liver. Hepatic radioactivity had to exceed 35 cpm per gram weight to utilize these calculations. Calculation of the minimum quantity of endotoxin detectable was based upon the assumptions that liver weight was 2 per cent of body weight, that 47 per cent of radioactivity localized in the liver following the portal vein injection of Cr^{51}-endotoxin, and that the LD_{50} for the dog was 150 mouse LD_{50} doses (Table V). The radioactivity necessary to exceed the counting error of 32 cpm or 10 cpm, depending on the counting interval, was calculated. The mean values and the range of minimum detectable quantities of endotoxin were based on the weights of the dogs employed; the range was 9.1 to 24.5 Kg. with a mean of 15.0 Kg.

RESULTS

A. Absorption of Cr^{51}-labeled endotoxin from the gastrointestinal tract during "irreversible" hemorrhagic shock

The levels of radioactivity in various tissues in control dogs, which received Cr^{51}-labeled endotoxin but were not bled, exceeded the counting error in only 1 of 13 dogs (Table VI, Group B).

TABLE VI

Absorption of radioactivity from the gastrointestinal tract of normal dogs and dogs during hemorrhage following the administration of chromic^{51} chloride

Group	Experimental preparation	No. animals	Animals with significant hepatic radioactivity (no./total)*
A	Control-free $Cr^{51}Cl_3$	7	6/7†
B	Control-Cr^{51} endotoxin	13	1/13‡
C	Shock-Cr^{51} endotoxin	21	4/21§

* Significant radioactivity is that which exceeds two standard deviations (S.D.) of the counting error of the sample and background. Background was 273 ± 2 cpm. The S.D. of the sample in most experiments was 12 to 14 cpm. In one dog in Group B and three dogs in Group C, the counting interval was increased so the S.D. of the sample was 5 cpm.

† In the dog which had insignificant radioactivity in the liver, significant though minimal radioactivity was present in the urine.

‡ In each dog in which significant radioactivity was detected in the liver, significant radioactivity was also detected in the urine.

§ The differences between Groups B and C are not significant (method of Chi square).

The calculated quantity of endotoxin absorbed was 0.0009 dog LD_{50}. The radioactivity in the urine of this group exceeded 32 cpm in three animals, including the dog with evidence of hepatic absorption. In eight of the nine animals receiving labeled endotoxin by gavage in Group B, the maximum level of radioactivity was found in the gastrointestinal tract below the pylorus. The maximum radioactivity was in the jejunum in three dogs, in the ileum in four dogs, and in the colon in two dogs. In those animals with enterostomies, three had cecostomies and one an ileostomy.

In dogs subjected to hemorrhage (Group C, Table VI) the radioactivity within the gastrointestinal tract was in the same range of activity as noted in the control animals. Descent of the radioactivity within the gastrointestinal tract following gavage was as follows: stomach, one dog; jejunum, two dogs; ileum, eight dogs; and colon, five dogs. Four shock animals had satisfactory cecostomies and one an ileostomy. Radioactivity was detected in the tissues of 4 of the 21 shocked dogs (Table VI, Group C). The estimated quantity of endotoxin absorbed by the shocked dogs was 1.35 mouse LD_{50} doses, 1.04 mouse LD_{50} doses, 0.04 mouse LD_{50} doses and 0.009 mouse LD_{50} doses. The two larger values were observed in the two dogs which had received Cr^{51}-labeled endotoxin 10.5 hours prior to the onset of hemorrhage and in both, significant levels of radioactivity were present in the urine at the time of sacrifice 15.5 hours after the administration of endotoxin, 129 and 57 cpm per ml. of urine. This radioactivity was probably the result of splitting off of some of the Cr^{51} from the Cr^{51}-labeled endotoxin in the gastrointestinal tract and excretion of free Cr^{51}. The radioactivity detected in the other two dogs was at the lower limit of detectability, though in one the sample was counted for 10 minutes.

Four of the shocked dogs died during the five hour period of hemorrhage; however, none had absorbed the minimal quantity of radioactivity from which endotoxin toxicity could be calculated.

B. Alteration in the susceptibility to endotoxin of dogs subjected to hemorrhage

Alterations in the susceptibility of dogs, either during or after "irreversible" or "reversible" hemorrhage, to the hypotensive and lethal effects of endotoxin were studied. The endotoxin administered during "irreversible" hemorrhage was a portion of Lot No. 3; however, this portion was not labeled with $Cr^{51}Cl_3$. The effect of the intravenous administration of sublethal quantities of endotoxin on the blood pressure of a normal dog is shown in Figure 1. This pattern of response is uniform and has been observed by other workers (24). Varying quantities of this same lot of endotoxin were administered intravenously to seven dogs during a period of protracted hypotension (five hours at 40 mm. Hg) and during the immediate post-reinfusion period when a temporary pressor response was evident. The first dose of endotoxin was administered after two hours of hemorrhage, then repeated and increased at each subsequent hour (Figure 2). Four of the seven dogs received at least 0.10 LD_{50} of endotoxin, while three of the seven received 0.10 LD_{50} both before and after reinfusion. No change in mean arterial blood pressure or loss of vasomotor tone as measured by an increased rate of "uptake" from the reservoir was noted in these animals. The response depicted in Figure 2 is representative of the seven animals. Even after reinfusion, the administration of 0.10 LD_{50} did not result in the immediate fall in mean arterial blood pressure that was observed in normal animals. A similar lack of effect of a single sublethal injection of endotoxin during hemorrhage in rats was observed by Zweifach and Thomas (16).

Susceptibility to endotoxin was studied after reinfusion of blood following a period of hypotension which in itself was nonlethal. The endotoxin used was Cr^{51}-labeled endotoxin in which the radioactivity had decayed, pooled from Lots No. 14, 15 and 17. The mouse toxicity of the pooled Cr^{51}-labeled endotoxin was again determined prior to injection. The Cr^{51}-labeled endotoxin was administered intravenously into a foreleg vein four hours after reinfusion of the shed blood.

In addition, estimated sublethal quantities of this Cr^{51}-labeled endotoxin were administered to normal dogs to be certain that the estimated doses were in fact sublethal (Table VII, Control Group). While the dosage was not lethal, the dogs which received an estimated 0.10 LD_{50} nevertheless developed symptoms of toxicity consisting of un-

TABLE VII

Susceptibility of dogs to the intravenous administration of endotoxin following hemorrhage of short duration ("reversible") *

Experimental procedure†	No. of animals	Duration of hemorrhage (*hrs.*)	Dosage of endotoxin‡ (dog LD_{50})	Mortality (no./total)
Control	5		0.01	0/5
	4		0.10	0/4
Reversible hemorrhage				
Group A	4	2	0	3/4
	4	2	0.01	4/4
Group B	3	1.5	0	1/3
	3	1.5	0.01	2/3
	6	1.5	0.001	1/6
Group C	2	1	0	0/2
	3	1	0.001	0/3

* Dogs were allowed to bleed to a mean arterial blood pressure of 40 mm. Hg and maintained at this level for periods of one or two hours, rather than five hours as in the "irreversible" shock group. The shed blood was reinfused after the shock period. Four hours after reinfusion, $Cr^{51}Cl_3$-endotoxin was injected into a foreleg vein. The mouse toxicity of the pooled $Cr^{51}Cl_3$-endotoxin was determined prior to injection.

† Control group consisted of normal dogs.

‡ The endotoxin dosage was based upon the assumption that one LD_{50} for the dog is 10 mouse LD_{50} doses per Kg. body weight. Control dogs received intravenous saline.

steadiness, collapse, wretching, vomiting and diarrhea.

Initially, a degree and duration of hypotension (40 mm. Hg for two hours) which has been "reversible" for other workers after reinfusion was used (18). This degree of hypotension was lethal in three of four control animals, so that an alteration in the degree of sensitivity to Cr^{51}-labeled endotoxin could not be ascertained (Table VII, Group A). Another group of animals was bled to a blood pressure of 40 mm. Hg for one and one-half hours (Table VII, Group B). This degree of hypotension was lethal for one of three control dogs. The administration of an estimated 0.01 LD_{50} of Cr^{51}-labeled endotoxin resulted in the death of two of the three shocked animals. The administration of an estimated 0.001 LD_{50} of Cr^{51}-labeled endotoxin to animals hypotensive for one and one-half hours was lethal for one of six dogs, while a similar amount of endotoxin was not lethal for three dogs maintained at 40 mm. Hg for one hour.

A number of the enterostomy procedures were not technically satisfactory for interpretation of absorption of labeled endotoxin because of leakage of radioactivity into the peritoneal cavity. However, these unsatisfactory experiments gave some further indication of the degree to which susceptibility to Cr^{51}-labeled endotoxin was altered in the dog during irreversible shock. In six shocked animals and in seven control animals radioactivity was present in the liver equivalent to the absorption of 0.13 to 65 mouse LD_{50} doses of endotoxin. Only two of the six shocked animals died; these two had absorbed smaller amounts of labeled endotoxin than other dogs in the group (1.0 and 1.3 mouse LD_{50} doses). None of the control animals which absorbed radioactivity equivalent to relatively small quantities of endotoxin (0.4 to 12.2 mouse LD_{50} doses) died during the period of study.

Thus, these experiments furnish no evidence of increased susceptibility to the repeated injection of endotoxin during shock or to the single injection of 0.001 LD_{50} of endotoxin following reversible hemorrhagic shock. Increased susceptibility to a single injection of 0.01 LD_{50} of endotoxin following a degree of hemorrhage, which in itself was occasionally lethal, was not clearly demonstrated.

DISCUSSION

Indirect evidence along several lines has implicated the importance of endotoxin from Gram-negative intestinal bacteria in the pathogenesis of "irreversibility" during prolonged hemorrhage (7, 10–12, 15, 16). Direct evidence to support this hypothesis has been dependent upon the development of techniques which permit the detection of endotoxin. The technique of isotopic

labeling of endotoxin with Cr^{51} ions seemed to be sufficiently sensitive to warrant its use in evaluation of the role of the absorption of endotoxin in "irreversible" shock. If endotoxin liberated from the bacterial flora within the lumen of the alimentary tract is an important factor in irreversible shock, either significant absorption of endotoxin from the gastrointestinal tract should be demonstrable during the development of "irreversibility," or a profound increase in the susceptibility of the shocked animal to amounts of endotoxin undetectable by Cr^{51}-labeling should be demonstrable.

Absorption of Cr^{51}-labeled endotoxin from various segments of the gastrointestinal tract, from the stomach to the colon, was not observed in 21 dogs subjected to a degree and duration of blood loss uniformly accepted as "irreversible" in at least 80 per cent of animals. Two of the animals were reinfused and absorption was not demonstrable during the pressor phase.

Likewise, if present, increased susceptibility to a single injection of endotoxin during the recovery phase of reversible hemorhage was in the range of 0.01 LD_{50}, which was well within the limits of detectability. Thus, the role of Boivin type bacterial endotoxin in the pathogenesis of the phenomenon of "irreversibility" following prolonged hemorrhage in dogs must be questioned.

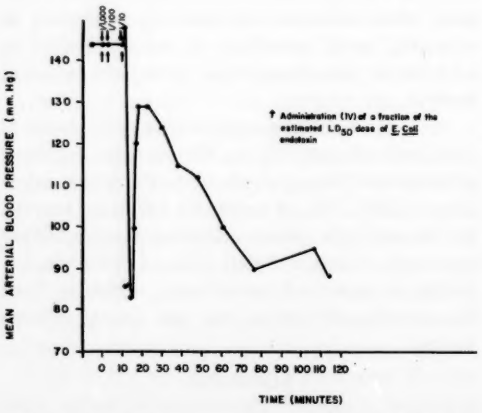

FIG. 1. THE EFFECT OF THE INTRAVENOUS ADMINISTRATION OF *ESCHERICHIA COLI* ENDOTOXIN IN VARYING QUANTITIES TO A NORMAL DOG

The fractions noted at each arrow represent a portion of the estimated LD_{50} for this dog. Following the administration of an estimated 0.10 LD_{50} or 15 mouse LD_{50} doses of unlabeled endotoxin an immediate fall in arterial blood pressure is noted.

These experiments might be criticized from several points. First, did the technique of labeling inactivate the endotoxin? Toxicity, as determined by lethality in mice, pyrogenicity in rabbits and the ability to produce shock and leukopenia in dogs, was demonstrated in various lots of endotoxin labeled using this technique. While six lots of labeled endotoxin were used in these studies, the quantity administered was determined on the basis of the mouse lethality of the labeled material.

Second, was the quantity of labeled endotoxin administered adequate? This varied from 53 to 133 mouse LD_{50} doses, with an average of 96 mouse LD_{50} doses. In conjunction with these experiments, studies on the quantitative bacterial flora of the gastrointestinal tract of normal dogs and of dogs subjected to "irreversible" shock were performed (25). The small intestine of the normal dog contained very few bacteria, approximately 1,000 per inch, of which two-thirds are Gram-positive, until the terminal six inches of ileum. The small intestine of animals subjected to "irreversible" shock contained approximately 45,000,000 viable aerobic bacteria per inch, of which Gram-negative organisms, especially *E. coli*, were predominant (25). The absolute quantity of endotoxin present in the gastrointestinal tract (80 inches) is difficult to determine. Landy and Johnson estimated that one billion organisms (Salmonella typhosa, 0–901) could contribute approximately 15 micrograms of lipopolysaccharide (endotoxin) with the LD_{50} for mice in the range of 250 μg. (21). From such rough approximations, the quantity of endotoxin administered definitely seemed adequate.

Third, did the Cr^{51}-labeling procedure alter the diffusibility of endotoxin across the gastrointestinal mucosa? This question is unanswered by the data. Likewise, the administration of barbiturate anesthesia in preparation for the shock procedure might have altered intestinal absorption. For this reason, sodium thiopental was used and the dogs were allowed to regain complete consciousness before starting the shock procedure.

Fourth, was the endotoxin administered representative? *Escherichia coli* is the predominant Gram-negative aerobic bacterium in the gastrointestinal tract of the dog; hence this bacterium was

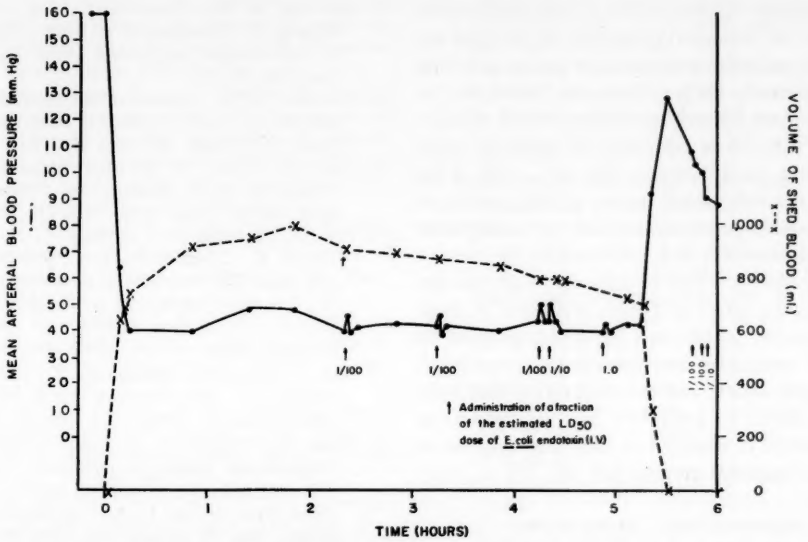
MEAN ARTERIAL BLOOD PRESSURE (mm. Hg)
VOLUME OF SHED BLOOD (ml.)
TIME (HOURS)
1/100
1/100
1/100 1/10
1.0
† Administration of a fraction of the estimated LD50 dose of E. coli endotoxin (I.V.)

FIG. 2. THE EFFECT OF THE INTRAVENOUS ADMINISTRATION OF A PORTION OF THE SAME LOT OF *ESCHERICHIA COLI* ENDOTOXIN AS DEPICTED IN FIGURE 1 IN VARYING QUANTITIES TO A DOG DURING A PERIOD OF PROLONGED HYPOTENSION AND AFTER REINFUSION OF THE SHED BLOOD

The solid line depicts the mean arterial blood pressure. The dotted line represents the volume of blood in the reservoir. The rate and volume of blood which has to be reinfused during the hemorrhage is a measure of vasomotor tone. Note the rapid decline in blood pressure following the reinfusion of the shed blood. This response is typical in the "irreversible" phase.

used as the source of endotoxin. Endotoxin derived from various species of bacteria demonstrates immunological specificity; however, the physiological activities of various lipopolysaccharide extracts are similar (26). While Boivin type endotoxin is the prototype used in most studies, there may be other toxic components in bacterial cells which are lost from the Boivin type of endotoxin.

Lastly, is the source of bacterial endotoxin from bacteria which invade the gastrointestinal mucosa and multiply outside of the intestinal lumen? Lillehei demonstrated that irreversibility was prevented in 90 per cent of dogs by perfusing the bowel with arterial blood from a donor dog during a five hour period of hemorrhagic shock, yet there was no difference in the bacteriologic findings between survivors and nonsurvivors (27). Lymph nodes were obtained from the coeliac and paracecal areas of three shocked animals which demonstrated extensive necrosis of the bowel mucosa (25). These were homogenized and cultured. The nodes contained fewer than 100 viable aerobic bacteria per node. Similarly, in a study of blood cultures obtained from normal dogs and dogs in hemorrhagic shock, no difference in the total percentage of positive cultures, or the percentage of Gram-negative organisms was found, (28). Thus, the number of bacteria present in the small intestine may increase during shock; however, they do not seem to invade the damaged gastrointestinal mucosa and multiply outside of the intestinal lumen.

Since absorption from the gastrointestinal tract of a Boivin endotoxin derived from *Escherichia coli* could not be demonstrated, the role of this classical type of bacterial endotoxin in the pathogenesis of the phenomenon of "irreversibility" following prolonged hemorrhage in dogs must either be questioned or further extra-intestinal sources of Gram-negative bacteria, including laboratory equipment, must be sought (29). Landy and

Shear obtained lipopolysaccharide materials which possessed the biological properties of bacterial endotoxin from various mammalian tissues including mouse stomach (30). Tolerance could be induced to these lipopolysaccharides which was associated with cross tolerance to bacterial endotoxin. One could postulate that as a result of the mucosal necrosis which occurs during prolonged hemorrhage, lipopolysaccharides of endogenous origin are absorbed and contribute to the genesis of irreversibility. This hypothesis would account for protective effect of bowel perfusion in dogs, the resistance which rats rendered tolerant to bacterial endotoxin demonstrate to hemorrhagic or traumatic shock, and the observation that hemorrhagic shock in germ-free rats is identical to that observed in normal rats including necrosis of the gastrointestinal mucosa (*27*, **17**, 31).

SUMMARY AND CONCLUSIONS

1. The possible role of absorption of endotoxin from the gastrointestinal tract in the genesis of the phenomenon of "irreversibility" in hemorrhagic shock was studied in dogs using endotoxin prepared according to the Boivin technique from a strain of *Escherichia coli* and labeled with $Cr^{51}Cl_3$. The technique was sensitive enough to detect approximately 0.001 of the LD_{50} dose of this endotoxin for normal dogs.

2. No absorption of Cr^{51}-labeled endotoxin from various portions of the bowel was observed in normal dogs or dogs subjected to "irreversible" hemorrhagic shock.

3. Increased susceptibility of dogs subjected to "reversible" hemorrhage and then injected with 0.001 LD_{50} dose of endotoxin was not demonstrated. If an increase in susceptibility did occur, it was in the range of 100-fold which was less than the minimal amounts of endotoxin detectable.

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GLUTATHIONE METABOLISM IN CORD AND NEWBORN INFANT BLOOD [1, 2]

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A hereditary abnormality of the erythrocytes was described in Negroes, sensitive to primaquine (1). A low level of reduced glutathione (GSH), a GSH instability, as well as a low activity of glucose 6-phosphate-dehydrogenase were demonstrated in those cells (2–5). A similar hereditary abnormality of the erythrocytes has been described in Jews originating from oriental and Mediterranean countries (6–10). It is associated with a susceptibility to hemolysis by various agents such as fava beans, sulpha drugs, para-amino salicylic acid and napthalene. Susceptibility to hemolysis by fava beans was also found in Italians of Sardinian origin and among Greek families residing in the United States (11–15). This hereditary abnormality is transmitted as a sex linked incompletely dominant trait with various degrees of expressivity of the abnormal gene ranging from full to nonpenetrance (9, 15). Zinkham and Childs recently described a nongenetic glutathione instability in the blood of normal newborn infants. This defect was associated with susceptibility to hemolysis by naphthalene as well as by vitamin K (14).

The present communication summarizes: *a*) studies on the level of GSH and on its stability in the erythrocytes of women during labor and in the umbilical cord blood, and *b*) further investigations on the mechanism of GSH instability in the erythrocytes of newborn infants.

MATERIALS AND METHODS

Blood used was obtained from *a*) women during labor, *b*) umbilical cord, *c*) 30 to 75 hour old infants, and *d*) eight day old infants. Controls were normal blood donors.

All subjects were divided into two ethnographic groups:

1. Ashkenazic Jews (originating from eastern, central and western Europe).

2. Non-Ashkenazic Jews (from oriental and Mediterranean countries).

The latter group was further subdivided according to the country of origin (9). A small number of Arabs was also included in the investigation.

GSH determinations. These determinations were performed on heparinized blood by the method of Grunert and Philips as modified by Beutler and associates (16, 1).

GSH stability test. The method of Beutler was used (2). Five mg. acetylphenylhydrazine per ml. blood or 0.02 mg. menadione sodium bisulfite (vitamin K) in saline was used. Previous investigations reported from this laboratory have demonstrated that when glucose is added to fresh blood the GSH stability test may be performed after 24 hours if blood is kept refrigerated without affecting the results (8, 9).

[1] Presented at the Conference on Enzymes in Blood, New York Academy of Sciences, Section of Biology, February 26–27, 1958.

[2] Aided by grants from the Israel Ministry of Health and the Blood Research Foundation, Washington, D. C.

TABLE I

Mean reduced glutathione (GSH) levels in normal and "sensitive" subjects*

	Normal subjects		Sensitive subjects	
	No. of subjects	Mean GSH*	No. of subjects	Mean GSH*
Cord blood	360	79.7 ± 13.1†	23	82.2 ± 13.4
Women during labor	269	69.6 ± 15.0	28	49.6 ± 10.4
Normal blood donors	219	70.4 ± 13.7	30	50.4 ± 13.5

* GSH in mg. per 100 ml. red blood cells.
† Mean plus or minus standard deviation.

TABLE II

Reduced glutathione (GSH) stability test

Origin of subjects	Sex	Control group*			Women during labor			Cord blood		
		No. of subjects	Sensitives		No. of subjects	Sensitives		No. of subjects	Sensitives	
			No.	*%*		*No.*	*%*		*No.*	*%*
Iraq	M	89	21	23				42	10	24
	F	91	22	24	72	20	28	33	6	18
Yemen	M	62	5	8				21		
	F	50	3	6	41	3	7	24	1	4
North Africa	M	43	1	2				40	1	2.5
	F	40	1	2.5	66	1	1	26		
Egypt	M	19						6	1	16
	F	9			14	1	7	6		
Turkey	M	22	2	9				21		
	F	22	1	5	34	2	6	15	1	6
Bulgaria	M	9						9		
	F	11	1	9	9			3		
Persia	M	9						10	1	10
	F	9	4	44	14	3	21	6	1	16
Others†	M	14	1‡	7				23		
	F	28	3§	10	37			18	1‖	5
Total non-Ashkenazic	M	267	30	11				172	13	8
	F	260	35	13	287	30	10	131	10	7
Arabs	M	40	1	2.5						
	F	13	1	8	3			2		
Ashkenazic	M	203						24		
	F	85			7			28		

* Three hundred four non-Ashkenazic and 288 Ashkenazic subjects summarized in a previous communication (9) are included.

† Including subjects from Greece, Buchara, Syria, Afghanistan and Sudan.

‡ Born in India, grandfather from Iraq.

§ Subjects from Sudan, Aden and one of mixed Greece-Buchara parentage.

‖ Parents from Greece.

The majority of determinations were performed with the addition of glucose (0.1 ml. 4 per cent glucose per ml. of blood). For some of the determinations fresh blood (*i.e.*, assay performed within two hours) without the addition of glucose was used. On the blood of infants a micro method was used. The amount of blood and reagents was reduced by a factor of five.

After deproteinization with 3 per cent metaphosphoric acid, aliquots were centrifuged for 10 minutes at 3,000 rpm in an international centrifuge. The optical density was read in a Klett-Summerson Colorimeter using a green filter and microtubes. The difference between duplicate estimations did not exceed 3 per cent. The results obtained agreed within 5 per cent with those obtained by the macro method.

The term "sensitive" was applied to individuals in whom the erythrocyte GSH level dropped below 30 mg. per 100 ml. red cells as a result of incubation with acetylphenylhydrazine or vitamin K.

Glucose-6-phosphate dehydrogenase activity was estimated. The amount of GSH formed in the following coupled reaction was determined:[3]

$$\text{G-6-P} + \text{TPN}^+ \xrightarrow[\text{dehydrogenase}]{\text{G-6-P}} \text{6-P-G} + \text{TPNH} + \text{H}^+$$

$$\text{GSSG} + \text{TPNH} + \text{H}^+ \xrightarrow[\text{reductase}]{\text{GSH}} 2\text{GSH} + \text{TPN}^+$$

The details of the technique were published elsewhere (10). Six-tenths mg. GSSG was added to a 3 ml. final reaction mixture. The activity of the dehydrogenase was estimated by the amount of GSH formed during a 15 minute incubation at room temperature.

[3] The following abbreviations are used: GSSG, oxidized glutathione; G6P, glucose-6-phosphate; 6PG, 6 phosphogluconate; TPN, triphosphopyridine nucleotide; TPNH, reduced triphosphopyridine nucleotide.

TABLE III

Tabulation of "sensitive" subjects according to the reduced glutathione (GSH) level after incubation with acetylphenylhydrazine

Source of blood	GSH level 0–10* Male	0–10* Female	11–20* Male	11–20* Female	21–30* Male	21–30* Female	Total Male	Total Female
Normal blood donors	7	3	4	5	2	9	13	17
Cord blood	5	2	7	3	1	5	13	10
Women during labor		3		10		15		28

* GSH in mg. per 100 ml. red blood cells.

Blood glucose was determined by the method of Hagedorn-Jensen (17).

RESULTS

GSH level in erythrocytes in cord blood, women during labor, and control group

These results are summarized in Table I. No difference in the GSH level was found between the control group (normal blood donors) and normal women during labor, while the mean GSH concentration in cord blood of normal infants was significantly higher ($p < 0.001$).

The mean GSH level in erythrocytes of "sensitive" individuals is known to be lower than the average value for normal subjects. However, in the cord blood no difference in the mean GSH level between "sensitive" and normal infant bloods was found. All subjects with GSH instability had normal GSH concentration in the blood obtained from the umbilical cord.

GSH stability test on bloods obtained from umbilical cord, women during labor, and control group

The results are summarized in Table II (592 subjects belonging to the control group and presented in an earlier publication are included in the table). No cases with GSH instability were found among the Ashkenazic subjects, while the mean frequency of GSH instability in the non-Ashkenazic groups was about 9 per cent. No significant differences were found in the frequency and ethnographic distribution of the erythrocyte GSH instability between cord blood and the other two investigated groups. In all three groups subjects originating from Iraq showed the highest frequency of the abnormality (above 20 per cent).

The observation that the GSH levels in "sensitive" males were usually lower than in "sensitive" females when their erythrocytes were incubated with acetylphenylhydrazine was found to be valid for the cord blood also (see Table III).

GSH stability in young infants

All tests presented in this section were performed by the micro method using fresh blood without the addition of glucose. The results are presented in Table IV.

All infants 30 to 75 hours old so far studied demonstrated a GSH instability, although cord blood from the same infants had stable GSH. The GSH instability was no longer demonstrable

TABLE IV

Reduced glutathione (GSH) stability in infants according to age

Source of blood	GSH* in fresh blood	GSH after incubation with APH†	GSH after incubation with Vitamin K
Cord blood			
With stable GSH	a) 90‡	82	80
	b) 89	56	60
	c) 83	60	56
	d) 67	56	58
With unstable GSH	84	11	15
	93	24	22
Infants, 30 to 75 hours old	a) 85‡	5	
	b) 90	6	
	c) 90	16	15
	d) 75	18	13
8 days old	84	51	60
	65	44	40
	102	65	

* GSH in mg. per 100 ml. red blood cells.
† Acetylphenylhydrazine.
‡ a, b, c, d, the same subjects examined at birth (cord blood) and at the age of 30 to 75 hours.

TABLE V

Influence of glucose and inosine on reduced glutathione (GSH) stability of blood obtained from umbilical cord, infants and adults

Source of blood	The fresh blood		GSH* after incubation with APH‡		
	GSH*	Glucose†	No glucose added	Glucose	Inosine
				4 mg./ml. blood	*5 mg./ml. blood*
GSH instability (hereditary)					
Cord blood	75	100	8	8	
	85	88	15	17	
	91	90	5	9	
Adult blood	38	117	8	8	7
	48	94	15	13	17
Infants (normal)					
30–75 hours old	90	79	16	80	
	75	65	15	60	
	80	69	20	89	92
	70		12	59	55
	102		19	98	80
8 days old	84	74	51	50	52
	65	79	44	70	54
	102		65	100	87

* GSH in mg. per 100 ml. red blood cells.
† Glucose in mg. per cent.
‡ Acetylphenylhydrazine.

in eight day old infants. The GSH instability was present in infants of Ashkenazic and non-Ashkenazic descent.

The effect of glucose and inosine on this GSH instability is summarized in Table V. Glucose as well as inosine have normalized the GSH instability in infants' blood, but had no effect on the hereditary type of GSH instability.

TABLE VI

Results of glucose-6-phosphate dehydrogenase assay *

Subjects	GSH (mg./3 ml. mixture) formed by reduction of GSSG
	mg.
Adults with stable GSH	0.25
	0.33
	0.38
Adults with GSH instability	0.04
	0.10
Infant 36 hours old	0.54
Infant 42 hours old	0.48
Cord blood with stable GSH	0.43
	0.40
Cord blood with GSH instability	0.02

* Incubation mixture contained 0.6 mg. GSSG.

This *in vitro* effect of glucose on the erythrocyte GSH instability in infants could be related to a lower glucose level in the newborn infants' blood. The glucose levels in the blood obtained from the umbilical cord, newborn and adult, are presented in Table V.

Although the glucose level in fresh blood obtained from 30 to 75 hour old infants was lower than in cord blood, the eight day old infants had blood glucose levels similar to those found in 30 to 75 hour old infants. Another explanation for the effect of glucose could be an increased utilization of glucose by the erythrocytes of the newborn. This is currently being investigated.

Glucose-6-phosphate dehydrogenase activity

In contrast to the hereditary type of GSH instability the enzyme activity in infant blood was not found to be decreased (see Table VI). These results confirm the data obtained by Gross and Hurwitz (18).

DISCUSSION

The results presented in this communication indicate that the hereditary abnormality of the erythrocytes which occurs in certain Jewish communities (and probably in other "races") manifests

itself already during fetal life. These cells demonstrate a GSH instability *in vitro,* a decreased G-6-P dehydrogenase activity and some reduction in GSH concentration. Recently an increase in glutathione reductase was demonstrated (19). Preliminary investigations in this laboratory indicate a decreased incorporation of glycine 1-C-14 into glutathione of "sensitive" erythrocytes *in vitro* (20).[4]

The mechanism of the *in vitro* erythrocyte GSH instability in 30 to 75 hour old infants is completely different from the inherited GSH instability. This erythrocyte GSH instability is not inherited nor is it racially determined. It appears only after birth and is very transient in nature. It is not accompanied by a decrease in G-6-P dehydrogenase activity. Furthermore, it may be prevented by raising the blood glucose or by the addition of inosine to the incubation medium. In this respect it is similar to the GSH instability appearing in normal blood in which the glucose content has been artificially lowered either by incubation for 24 hours at room temperature or by suspending washed erythrocytes in saline or buffer solution devoid of glucose (2, 3, 8). Glycine 1-C-14 incorporation into the erythrocyte glutathione of those newborns is normal, while in the hereditary abnormal erythrocyte the glycine incorporation is low (20).[4] The glucose level in the blood of young infants did not differ much from that found in cord blood or eight day old infants' blood. In spite of these *in vitro* results, the effect of low blood glucose concentration on GSH instability in newborns cannot be completely discarded at present. The physiological hypoglycemia is generally more pronounced and of longer duration in prematures than in normal termed infants (21, 22). Most hemolytic reactions due to vitamin K administration have been described in premature infants (23, 24). Hemolysis following naphthalene ingestion or unidentified agents in such infants is also known (25, 26). It is even possible that such drugs administered to nursing mothers could be transmitted through the milk to the infants and cause hemolysis in the infant in analogy to cases of favism caused by milk of goats fed fava beans as mentioned by Luisada (27).

The mechanism by which glucose or inosine exhibit a protective effect upon the erythrocyte GSH instability is not clear.

Glucose probably enters the cell via an active process which is catalyzed by hexokinase whose coenzyme is adenosine triphosphate (ATP). The glucose is further metabolized via the glycolytic pathway and probably to some extent via the hexose monophosphate shunt (28, 29). Beck has shown that the extent to which white blood cells metabolize glucose via the shunt mechanism is directly determined by the glucose-6-phosphate level in the cells and indirectly by the hexokinase activity (30). It is possible that a similar mechanism operates in the erythrocytes also.

The hexokinase activity and the ATP level in the red cell of the newborn infants is under investigation.

The normal GSH stability in cord blood in comparison with its instability during the first days of the infant's life could be the result of an increased permeability of glucose into the fetal red cells *in utero.* Those possibilities are being further investigated.

SUMMARY

The reduced glutathione level and its stability has been studied in women during labor and in cord blood. The mean glutathione level in cord blood was found to be higher than in adult bloods. The hereditary abnormality of the erythrocyte leading to susceptibility to hemolysis was already detectable in cord blood. In the Jewish population, this abnormality was present in non-Ashkenazics only, the highest frequency being encountered among those of Iraqi origin. The reduced glutathione level in cord blood of "sensitive" subjects was within normal limits.

In normal newborn infants a transient, nonhereditary glutathione instability appears during the first hours of life. It is not associated with defective glucose-6-phosphate dehydrogenase activity. This erythrocyte reduced glutathione instability may be prevented by the addition of glucose or of inosine to the blood *in vitro.* The pos-

[4] Additional investigations of glycine 1-C-14 incorporation rate into erythrocyte glutathione (GSH) suggested that the low specific activity of GSH isolated from the "sensitive" erythrocytes could be caused not by low glycine incorporation rate, but by the rapid disappearance of GSH from these cells due to the presence of cystein in the incubation mixture. The experimental details will be published.

sible mechanism of the nonhereditary reduced glutathione instability is discussed.

ACKNOWLEDGMENT

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THE USE OF RADIOIODINATED ALBUMIN IN METABOLIC STUDIES. THE EFFECTS OF THE LEVEL OF DIETARY PROTEIN AND L-TRIIODOTHYRONINE ON THE CATABOLISM OF RADIOIODINATED HUMAN SERUM ALBUMIN [1]

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Radioiodinated proteins have been widely used as a convenient tag for plasma proteins since the description by Sterling (1). Recently evidence has been summarized suggesting that in many studies iodinated proteins do not behave as does the native protein (2). The chemical mixture for attaching the iodine label, the number of iodine molecules per protein molecule and the irradiation arising from the label may affect the protein molecule. Thus, proteins iodinated in most laboratories disappear from the plasma more rapidly than protein containing endogenous labels of N, C or S (3–6). This difference is decreased by the use of carefully prepared iodinated proteins (7, 8) and is probably accounted for on the one hand by denaturation of the iodinated protein (7–9) and on the other hand by slight reutilization of the N, C or S label (3, 4, 6).

This paper presents a modified method of using human iodinated serum albumin (HISA), commercially prepared, in which the same preparation is employed in both a control and an experimental period. It will be shown that high levels of dietary protein and the administration of large doses of L-triiodothyronine increase the catabolic rate of HISA.

METHODS

Subjects, diets, and collections. All subjects were confined to a metabolic ward throughout the period of study. Liquid diets as previously described (10) were administered in divided feedings. Table I gives pertinent clinical data on the subjects and lists the calculated protein, fat, carbohydrate and caloric content of the diets during each study. An equilibration period of at least three days on the diet was employed before collections were begun. All subjects (except No. 7) received 0.2 ml. Lugol's solution three times daily while under study. Urine and feces were stored under refrigeration throughout the collection period. Collection periods were of 24 hours duration for urine and of 72 or 96 hours duration for feces. Urinary creatinine was determined on all urine specimens as a check on the completeness of collection. The methods of chemical analysis are those previously described (11).

Labeling of the patient, analysis for radioactivity. HISA was administered intravenously within two days of receipt of the shipment from the manufacturer.[7] All lots of HISA were dialyzed to determine the fraction of the radioactivity unbound to protein. All lots employed in these studies had less than 3 per cent dialyzable radioactivity. Approximately 100 μc. was employed for each study of 16 days duration; the dose was doubled for each 8 additional days planned but did not excede 400 μc. The calculated dose was taken up in a tared tuberculin syringe which was then reweighed. The HISA was given intravenously and the syringe was rinsed three times with the patient's blood while still in place, giving the rinsed blood back to the patient. A standard HISA solution was simultaneously prepared by rinsing the contents of a similar syringe into a volumetric flask employing three rinses with water rather than blood. A Tracer Laboratories well scintillation counter Model DS3 with a Nuclear number 183 scaler was used for counting. Counting periods were in general three minutes but were prolonged when necessary to measure a minimum of 1,000 counts above background.

[1] An abstract of this article appeared in J. clin. Invest. 1956, 35, 705.

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[7] Abbott Laboratories radioiodinated serum albumin (RISA®) was used throughout these studies.

TABLE I

Pertinent clinical data and dietary intake on each experimental subject

No.	Age	Sex	Diagnosis	Study	Wt.	Protein	CHO	Fat	Calories/day
	yrs.				*Kg.*	*Gm./day*	*Gm./day*	*Gm./day*	
1	20	M	Herniated nucleus pulposis (HNP), 6 weeks postoperative	High protein	97	292	279	125	3,420
				Low protein		50	402	179	3,390
2	20	M	Contracting scars on hand	High protein	87	265	253	113	3,100
				Low protein		44	269	204	3,040
3	60	M	Senile osteoporosis	High protein	48	149	195	42	1,760
				Low protein		26	207	92	1,740
				Triiodothyronine (T-3)		149	195	42	1,760
4	25	M	Adrenalectomized for Cushing's disease; maintained 50 mg. hydrocortisone; 6 months postoperative	High protein	72	219	236	81	2,540
				Low protein		41	352	112	2,560
				T-3		62	66	22	710
5	26	M	HNP, 8 weeks postoperative	High protein	89	265	255	111	3,070
				Low protein		46	380	168	3,190
				T-3		46	380	168	3,190
6	27	M	Obesity and eosinophilic granuloma of lung and bone irradiated 5 months previously	T-3	100	82	60	7	620
7	22	M	Total thyroidectomy for papillary carcinoma, 4 months postoperative, maintained on 3 grains thyroid per day	Lugol's solution	102	102	502	181	4,020

Blood samples were drawn 20 minutes after the labeling dose was given and each morning thereafter, using dry heparin as the anticoagulant. Duplicate 1 ml. samples of plasma were pipetted into counting tubes and held for counting. Duplicate 1 ml. samples of urine were taken from each 24 hour urine collection; two successive 24 hour collections were then combined and aliquots taken for creatinine and nitrogen analysis. When the radioactivity of the urine specimen was too low for accurate counting, concentration of radioactivity was carried out as follows: 10 ml. of urine was placed directly in a counting tube and 0.2 ml. of 0.1 N sodium thiosulfate, 1 ml. of 10 per cent nitric acid and 2 ml. of 10 per cent silver nitrate were added. The tubes were heated in a boiling water bath for 15 minutes and after cooling were centrifuged to concentrate the precipitated AgI^{131} in the bottom of the tube where it could be counted without removing the supernatant. Pure solutions of NaI^{131} were totally recovered by this procedure and in urine at least 97 per cent of the radioactivity was recovered (average of 10 urines, 98.8 per cent).

Presentation and interpretation of data. The experimental data are presented as outlined by Berson, Yalow, Schreiber and Post (8). The radioactivity of the plasma samples was plotted as a function of time on semilog. paper; this will be referred to as the "plasma curve." The radioactivity in the urine was also plotted as a function of time on semilog. paper, but it was soon discovered that failure to empty the urinary bladder completely at the end of each collection period introduced considerable fluctuation in many of the urinary curves. For this reason, the urinary excretion data are presented in the per cent remaining curve, a semilog. plot of the radioactivity that remains within the body at any given time plotted as a function of time. Day by day fluctuations are minimal in the per cent remaining curve, rendering it much more susceptible to statistical calculations.

The plasma curve is influenced by one or more of the following: *a*) changes in plasma albumin concentration or total albumin mass, *b*) the disappearance of intact HISA or its labeled breakdown products from the plasma, *c*) the synthesis of new albumin, and *d*) the catabolism of HISA. If *a*) does not change (apart from new synthesis) and the labeled breakdown products of HISA disappear promptly from the plasma, the plasma curve is then a measure of the rate of synthesis and the rate of catabolism of albumin. Similarly, the per cent remaining curve is influenced by *a*) the excretion of HISA or of its labeled breakdown products (other than I^{131}), *b*) the excretion of I^{131} and *c*) the catabolism of HISA. If the only significant product of HISA is labeled iodide and the I^{131} is excreted promptly, the per cent remaining curve is then a measure of the catabolic rate of albumin. The validity of these assumptions has been considered by Berson and co-workers (8).

None of the present patients had proteinuria. Occasional T-1824 spaces in each patient and daily measure of the serum protein showed no significant change dur-

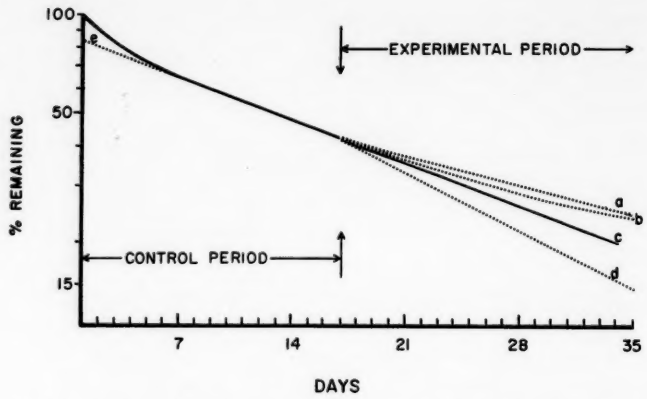

FIG. 1. HYPOTHETICAL PER CENT REMAINING CURVE SHOWING THE ALIGNMENT OF A CONTROL PERIOD AND AN EXPERIMENTAL PERIOD

Lines a, b and d represent possible variations from line c obtained by extrapolation of the linear portion of the control period.

ing the studies. In the calculation of the per cent remaining curve, correction was made each day for the I^{131} removed in the blood sample. When the per cent remaining is 20 per cent or less, this curve becomes exquisitely sensitive to both slight losses of urine and errors in the dose given; for this reason, these factors were carefully controlled.

In the present experiments sufficient HISA was given so that a single label would extend throughout two periods, a control and an experimental one. Figure 1 presents a hypothetical curve to illustrate experimental design. Curve c represents an extension of the best line observed during the control period. It has been demonstrated (8) that such curves tend to become more and more flattened as observation is prolonged, as illustrated in Figure 1, Curve b—they never become steeper. If a variable were introduced at the end of the control period, resulting in a decreased slope (Curve a) the change could not be distinguished from that frequently observed spontaneously. If, however, the control and experimental periods were so aligned that an increased slope occurred during the experimental period (Curve d), the significance of the change would be indisputable. In this event, the magnitude of the change may be estimated by subtracting the per cent change per day [8] of the control curve (c) from that of the experimental curve (d) and multiplying this difference by the total exchangeable albumin (8) in grams. This yields a *minimal* estimate of the increased breakdown of albumin in grams per day, for the extrapolated control curve, had it remained undisturbed, might have gradually flattened (Curve b).

[8] The half-life ($t_{1/2}$) is the time necessary for the plasma radioactivity curve (or the per cent remaining curve) to reach ½ of its initial value and is obtained graphically. The per cent change per day is calculated from the formula, $\ln 2/t_{1/2} \times 100$.

The statistical calculation in each instance is to compare the slope of the line on the left half of the graph with the slope of the line on the right half of the graph (12). In these calculations, as employed later, the first six points on the graph are arbitrarily omitted from consideration since this number of points seems to include the necessary time for the equilibration of the HISA with the extravascular albumin pool. Unless qualified, "significant" means significant at the 1 per cent level.

Influence of protein content of the diet—Experimental and results. Five subjects were studied (Table I, Subjects 1 through 5). Two isocaloric diets were employed in the study of each subject, one containing 0.5 gram and the other 3.0 grams of protein per Kg. of body weight. A representative study is illustrated in Figure 2. After the subject had equilibrated to the low protein diet (not shown in Figure 2), he was given HISA and the plasma levels and urinary excretion of radioactivity were measured for two weeks. The diet was then abruptly changed on Day 15 to high protein content and the radioactivity measurements were continued long enough to detect any change in the slope of the curves (Days 15 to 21). A second dose of HISA [9] was then given (Day 21) while the subject remained on the high protein diet. After the new curves were established (Days 21 to 35) the diet was abruptly returned to the low protein (Day 36). The first per cent remaining curve in Figure 2 shows more rapid catabolism of HISA when the protein intake is

[9] If appreciable plasma radioactivity were left from the previous dose of HISA, the size of the second dose was increased to the maximum permissible to lessen the influence of a different lot of HISA. The "total dose" employed in the calculation of the per cent remaining was the amount given plus the amount still present in the body. The latter was estimated from the plasma activity and the measured pool size.

suddenly shifted from low to high. Similarly, the second per cent remaining curve in Figure 2 shows a flattening when the dietary protein intake is suddenly dropped from high to low; unlike the first curve, however, this does not necessarily indicate a change in albumin metabolism since the same change in the curve might have been seen without any change in the experimental situation (see Figure 1 and the discussion of Figure 1).

The dietary protocols for three of the five subjects were identical to that illustrated in Figure 2; in the other two subjects, the sequence was reversed, the diets being high protein, low protein and high protein in that order. The composite experience of all five subjects is shown in Figures 3 and 4. In four of the five studies there was significantly increased catabolism of HISA when the dietary protein was suddenly increased (Figure 3). Although three of the five studies showed a statistically significant decrease in slope of the per cent remaining curve when protein intake was suddenly decreased (Figure 4), the normal flattening of HISA curves (see Figure 1) does not permit interpretations of changes in this direction. The scatter observed in the plasma values was so great that the changes were not interpretable (sample data, Figure 2).

Discussion. Steinbock and Tarver, in studies using S^{35} labeled amino acids in rats, found that the half-life of total serum protein was 17 days in animals eating a protein free diet, 5.1 days in animals on a diet containing 25 per cent protein and 2.9 days in animals on a diet containing 65 per cent protein (13). Liver tissue removed from animals maintained on a high protein diet incorporated amino acids more rapidly than liver from animals on a low protein diet (14). Blahd, Fields and Goldman (15) measured the effect of diet on albumin turnover in patients with the nephrotic syndrome and found that an increased level of dietary protein accelerated the albumin synthesis.

The present study shows that the catabolism of HISA

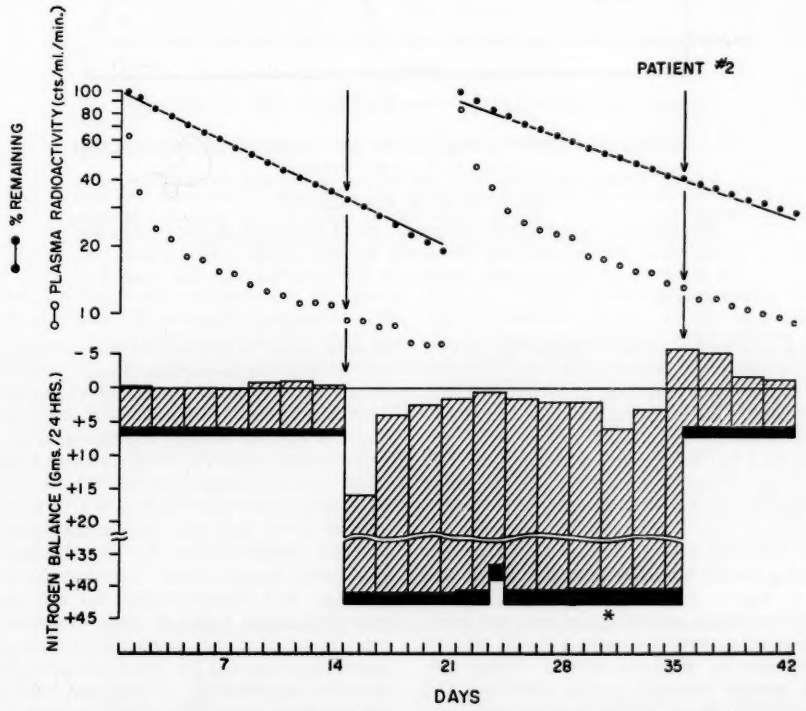

FIG. 2. EFFECT OF VARYING PROTEIN INTAKE ON ALBUMIN TURNOVER

The diet was changed from 0.5 Gm. protein to 3 Gm. protein per Kg. body weight. The straight lines in the per cent remaining curves are extrapolated from the early portion (Days 1 through 14) and (Days 21 through 35) into the later portion. From Days 14 through 21 the experimental points drop below the line indicating increased catabolism. Plasma radioactivity has been divided by 10^4. Asterisk indicates patient had one meal of solid food—part of usual diet removed to keep intake constant. The scale for the plasma values is arbitrarily chosen.

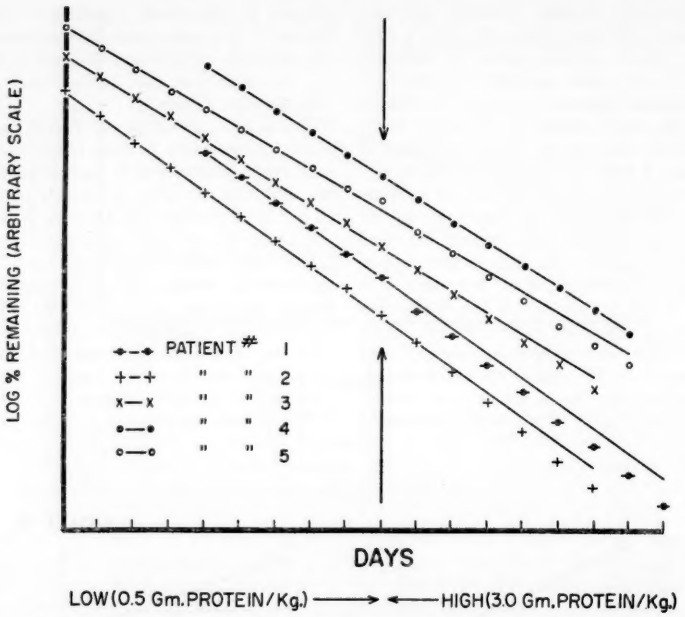

FIG. 3. EFFECT OF *INCREASING* THE INTAKE OF PROTEIN ON THE RATE OF DEGRADATION OF RADIOALBUMIN

The lines represent the least square fit line of the period of low protein administration extrapolated into the period of high protein administration. In all except No. 4 the points fall beneath the line indicating increased catabolism of albumin.

is accelerated by a sudden surfeit of protein in the diet. The maximum change was seen in Subject 1 in whom the half-life of the per cent remaining curve changed from 11.5 to 9.5 days. This represents an increased breakdown of approximately 1.2 grams of albumin per day. The albumin pool was 350 grams and this increase was 17 per cent in the per cent change per day. In addition to using the increased amounts of dietary protein for energy metabolism and protein stores (note the positive nitrogen balance), the body seems to increase the replacement rate of existing protein molecules; the lack of response in Subject 4 (Figures 3 and 4) is interesting, for this man had no functioning adrenal tissue and was maintained entirely on exogenous compound F (50 mg. per day). Administration of excess adrenal cortical hormones may produce increased protein catabolism (16, 17). The catabolic effect of sudden increases in the protein in the diet may therefore be mediated through the adrenal cortical hormones. It is possible that the prolonged survival of plasma proteins tagged with S^{35} observed by Armstrong and associates (18, 19) in uremia was due to relative protein starvation, if this were prescribed as part of treatment of the disease.

The diets used in this study were isocaloric and therefore increase in the protein content was accompanied by decrease in the fat and carbohydrate content. The possible effect of the concomitant changes in fat and carbohydrate intake on HISA catabolism was not investigated.

Influence of L-triiodothyroinine—Experimental and results. The effect of the acute administration of large doses of L-triiodothyronine on HISA catabolism was studied in four subjects (Table I, Subjects 3 to 6). A representative study is illustrated in Figure 5. The subject was observed for 14 days after the administration of HISA while the plasma and the per cent remaining radioactivity curves became stable. Five hundred micrograms (0.5 mg.) of L-triiodothyronine was then administered every 12 hours for 7 days [10] (Days 15 through 21), and measurement of the urine and plasma radioactivity was continued during, and for two weeks after, the triiodothyronine administration. A composite chart of the per cent remaining curves from all four subjects is presented in Figure 6. In all four curves there is a significant increase in the slope of the curve which corresponds in time to the period of negative nitrogen balance. Table II presents data derived from the experiments in each subject. The cumulative negative nitrogen balance during

[10] In one experiment, Subject 4, triiodothyronine administration lasted only four days.

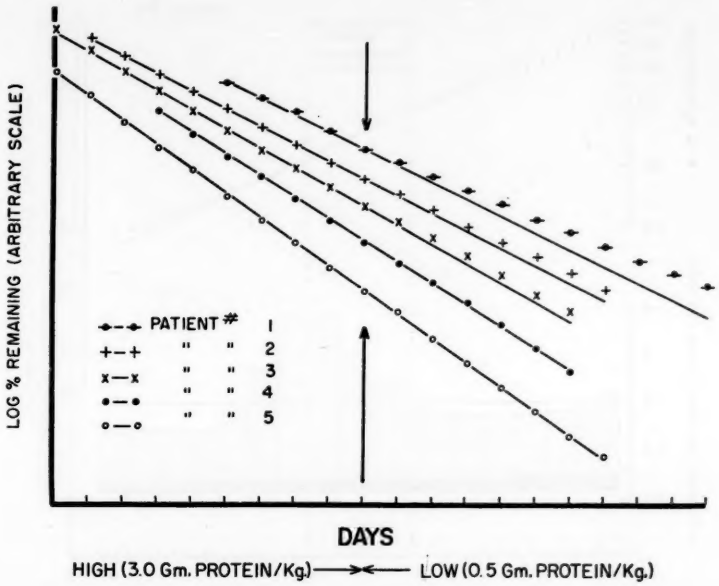

FIG. 4. EFFECT OF *DECREASING* THE INTAKE OF PROTEIN ON THE RATE OF DEGRADATION OF RADIOALBUMIN

The lines represent the best fitted line of the period of high protein administration extrapolated into the period of low protein administration. In all except Patients 4 and 5 the points fall above the line. The meaning of this change is uncertain.

the period of triiodothyronine administration, the size of the albumin pool and the percentage breakdown of the serum albumin per day, before and during triiodothyronine administration, are noted. The cumulative negative nitrogen balance was calculated from a baseline established during the control period and arbitrarily set as zero. This "negative" balance thus represents increased whole body nitrogen catabolism. Subjects 3 and 5 were on a diet containing adequate calories (approximately 35 cal. per Kg.), whereas Subjects 4 and 6 were on deficient caloric intake; the latter pair showed a much greater increase in albumin catabolism during the period of triiodothyronine administration than the subjects on adequate calories. In the two on deficient diets, the increased catabolism of serum albumin was equivalent to 69 and 112 per cent of the cumulative negative nitrogen balance. In the two subjects on normal caloric intake, the increased catabolism of serum albumin was equivalent to 10 and 21 per cent of the negative nitrogen balance (see Table II). The average ratio of albumin nitrogen to total body nitrogen was 2.2 per cent (Column 6, Table II). In these studies no fall in the serum albumin was observed; therefore, it is likely that albumin anabolism increased and unlikely that the increased

TABLE II

Effect of triiodothyronine (T-3) on albumin catabolism and nitrogen metabolism

Subject	(1) T-3 duration	(2) Cumulative negative N balance	(3) Estimated total body N (Ref. 20)	(4) Albumin pool Alb.	(5) Albumin pool N	(6) $\frac{\text{Alb. N}}{\text{Total N}}$ $\frac{(5)}{(3)} \times 100$	(7) % Breakdown *alb./day* Before T-3	(8) % Breakdown *alb./day* During T-3	(9) N due to increased alb. breakdown $\frac{(8)-(7)\times(1)\times(5)}{100}$	(10) $\frac{(9)}{(2)} \times 100$
no.	*days*	*Gm.*	*Kg.*	*Gm.*	*Gm.*	%			*Gm./day*	%
3	7	17.3	1.31	224	35.9	2.7	3.77	4.5	1.8	10
4	4	13.3	1.95	255	40.8	2.1	7.30	12 2	8.0	60
5	7	31.3	2.42	302	48.3	2.0	6.35	8.3	6.6	21
6	7	21.7	2.71	367	58.7	2.2	8.16	14.1	24.3	112

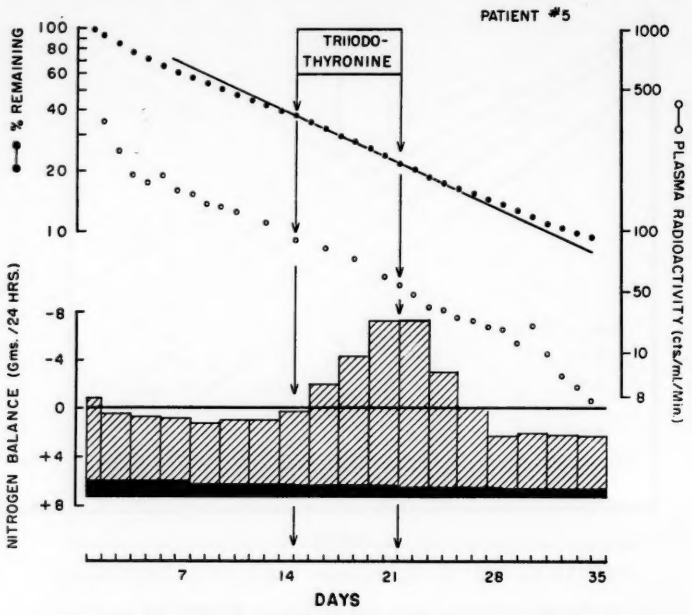

FIG. 5. EFFECT OF TRIIODOTHYRONINE ON ALBUMIN TURNOVER

The line in the per cent remaining curve is the best fitted line to the points during triiodothyronine administration. Notice that the points in the precontrol period fall below this line and the points in the postcontrol period fall above it. In both, the rate of catabolism is slower than during triiodothyronine administration. The triiodothyronine effect persists longer than its time of administration, as indicated by the prolonged negative N balance. The plasma values have been divided by 1,000.

albumin catabolism actually accounts for much of the negative nitrogen balance.

Discussion. Schwartz (21) reported that the half-life of HISA was increased in myxedema and that restoration of the euthyroid state was associated with a return of the biological half-life toward normal. These findings were confirmed by Lewallen, Rall, Berman and Hamel (22). Dixon, Maurer and Deichmiller (23) reported a

TABLE III

Comparison of triiodothyronine (T-3) data

	Crispell and associates (24)	Rothschild and associates (25)	Present study
Technique	N^{15} glycine tag	Iodinated albumin	Iodinated albumin
Dose thyroid	0.105 mg./day T-3	12–18 grains desiccated thyroid/day	1 mg./day T-3
Clinical change	None	Marked thyrotoxicosis	Marked thyrotoxicosis
Neg. N balance	Slight, av. 1 Gm./day	Not measured	Marked, av. 5 Gm. day
Serum protein		Slight fall	No change
Albumin pool		Fell	Not measured
Plasma vol.		No change	No change
Conclusion	Algebraic average of protein synthesis is slowed	Serum albumin catabolism is increased; serum albumin synthesis increased	Serum albumin catabolism is increased
Deduction	Anti-anabolism is cause of negative nitrogen balance		Accelerated catabolism is cause of negative nitrogen balance; albumin synthesis increased to maintain serum protein

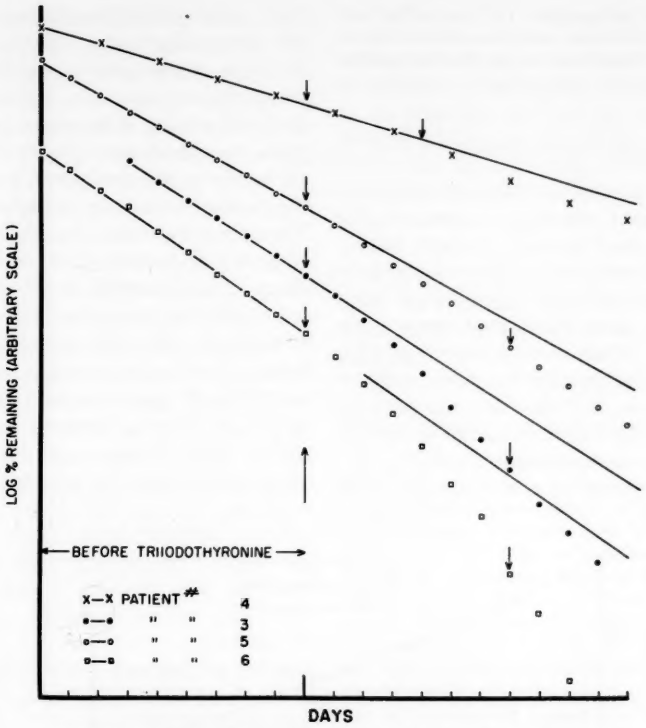

FIG. 6. EFFECT OF TRIIODOTHYRONINE ON ALBUMIN TURNOVER

The arrows delineate the duration of triiodothyronine administration. The lines are the best fit during the pretriiodothyronine control period. Note the apparent persistence of triiodothyronine effect on the curves after cessation of its administration.

decreased half-life of labeled albumin in normal rabbits treated with thyroxine. Crispell, Parson and Hollifield (24) studied the incorporation of N^{15} labeled glycine into the body proteins in patients with primary myxedema and found that soon after the administration of minimal doses of triiodothyronine the rate of protein synthesis decreased, but at higher doses the rate increased toward normal. In a normal subject, small doses of triiodothyronine produced a definite decrease in the rate of protein synthesis.

Rothschild, Bauman, Yalow and Berson (25) have presented studies similar to the present ones in euthyroid subjects receiving 12 to 18 grains of desiccated thyroid daily. They found increased catabolism of serum albumin. The present study shows about twice the rate of catabolism during the control period as compared to Rothschild and co-workers' values, reflecting differences in the iodinated albumin employed. Rothschild and associates reported that the mean increased catabolism during thyroid administration was 30.3 per cent; the two subjects in the present study on normal diets showed values of 28 and 30 per cent. The two subjects on caloric deficient diets showed 60 and 75 per cent. Rothschild and associates found a decline in the serum albumin concentration and a fall in the total exchangeable albumin, but the latter fall was not as great as the increased catabolic rate would produce during the period of observation so that synthesis was increased. In our study a change in albumin concentration was not found and the plasma volume (T-1824) did not change. The similarities and the differences in the experimental studies of Crispell and co-workers (24), Rothschild and co-workers (25) and the present authors are presented in Table III, along with the conclusions and deductions that may be drawn.

From this it seems likely that thyroid hormone acts similarly in normal subjects receiving large doses and in myxedema subjects receiving replacement therapy. Both catabolism and anabolism of serum albumin are increased and negative nitrogen balance results, at least during the early administration. Anabolism of serum albumin is increased almost to the same degree that catabolism is increased. The lower dose of triiodothyronine

employed by Crispell and associates (24) in a normal subject to demonstrate decreased anabolism was not used in the present study. The plasma values showed too great a scatter for statistically valid conclusions regarding albumin anabolism.

GENERAL DISCUSSION

HISA as it is commonly prepared gives variable plasma and per cent remaining curves, and this considerable biological variation has been emphasized (8). This variation is observed when the same lot is given to different individuals or when different lots are given at different times to the same individual. It has recently become possible to prepare iodinated proteins that have more reproducible half-lives, but these are not generally available (7). The HISA preparation used in this study has been compared with HISA produced by the more recent methods (26). The HISA employed in these studies demonstrates this lack of reproducibility. Thus in Figures 3 and 4, the slopes of the curves on the lefthand half of the charts are not identical. Similarly, in Figure 2, the initial portion of the second per cent remaining curve has a much more shallow slope than the initial portion of the first curve, despite the fact that the patient is now on a higher protein intake. Thus, the comparison of moderate changes in the slope of HISA curves between individuals or between sequential studies in the same subject at different points in the course of diseases is not valid. It has been shown that a single HISA label will follow a predictable pattern (see discussion, Figure 1) for 35 days or more (Figure 7). By use of a single label for both the control and experimental study, comparisons become valid. This comparison holds even though HISA may not be

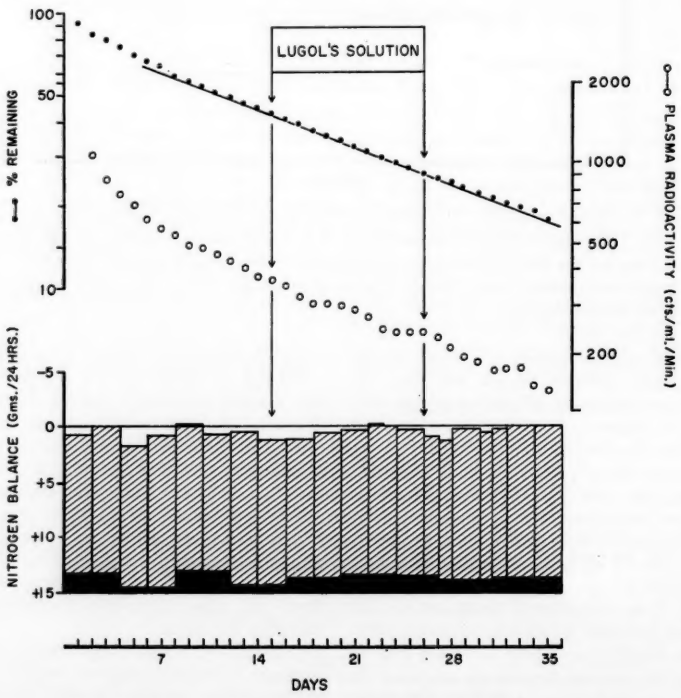

FIG. 7. EFFECT OF LUGOL'S SOLUTION ON ALBUMIN TURNOVER

The line in the per cent remaining curve is the best fitted line to the points during the administration of Lugol's solution. The points in the precontrol and postcontrol period do not differ significantly from this line. The nitrogen balance did not change during the administration of Lugol's solution.

a perfect tracer for serum albumin. This technique was used by Lewallen and co-workers (22) prior to the first report of these studies (27).

The use of samples of plasma to establish the turnover curve has several deficiencies that are largely corrected by use of the cumulative urinary excretion or the per cent remaining curve. A plasma sample represents a variable daily aliquot of the total albumin pool subject to fluctuations of hydration. The cumulative urinary excretion, on the other hand, gives a sensitive daily measure of change in the entire albumin pool (28). The curve obtained from the plasma data is influenced by both anabolism and catabolism of the plasma proteins. The cumulative urinary excretion is a function only of catabolism.

An isotopic label that is significantly reincorporated into proteins cannot be used for such studies as these, for all of the findings could represent alteration in the rate of reincorporation. Sterling (1) has reviewed the overwhelming evidence that the radioactive label of iodinated proteins is not reincorporated into newly synthesized native proteins. Margen and Tarver (6) suggest that *in vivo* exchange of I^{127} with the radioactive I^{131} may occur and thus lead to a spuriously short life of iodinated proteins. To test such a possibility, Lugol's solution was administered to an athyroid subject after an HISA per cent remaining curve had been established. This administration did not alter the catabolic rate of HISA (Appendix and Figure 7). If such a mechanism as proposed by Margen and Tarver had been of measurable importance, increase in the apparent catabolism of HISA would have been expected.

In the present interpretation of the per cent remaining curve, it is assumed that the excretion of I^{131} from the plasma into the urine remained constant. Thyroxine (29) increases renal blood flow. Could this change account for the experimental observations? The excretion of I^{131} is exceedingly rapid compared with the rate of breakdown of HISA (8) and, therefore, any increase in the renal clearance of I^{131} would manifest itself by an increased urinary excretion of I^{131} for one or two days, but this excretion would then return to the slower rate determined by the release of I^{131} from the catabolism of HISA. Such a possibility therefore seems unlikely.

SUMMARY

Human iodinated serum albumin was employed as a label of plasma albumin. A single injection of the label was used for both the control and experimental observations. Observations were made for periods up to 35 days. Very high protein intakes were found to accelerate the catabolism of serum albumin significantly over the values observed when the same subjects received isocaloric diets lower in protein. Triiodothyronine accelerated the catabolism of serum albumin simultaneously with the production of negative nitrogen balance.

APPENDIX

Effect of Lugol's solution on HISA curves

A male subject surgically rendered athyroid was studied some four months after surgery. The experimental design (Figure 7) was similar to that used for triiodothyronine except that he was not given Lugol's solution initially, for there was no need to block the thyroid. During the period from Day 15 to 26, 0.2 ml. of Lugol's solution was administered orally three times daily. There was no significant change in the N balance, and the experimental points in both the precontrol and postcontrol period on the per cent remaining curve do not deviate significantly from the line defined during the administration of Lugol's solution. See text for discussion.

ACKNOWLEDGMENT

Captain Patty Shuman and her staff provided nursing care and continuous observations during these studies. The meticulous care of the dietary and laboratory staff at the Walter Reed Metabolic Ward was responsible for the precision of the data. Miss Marjorie Knowlton aided in all phases of the study. Dr. Glenn Bartsch provided assistance with the statistics. The cooperation of the seven volunteer subjects who willingly ingested liquid diets and permitted innumerable venipunctures is gratefully acknowledged.

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STUDIES OF RIBOSE METABOLISM. V. FACTORS INFLUENCING *IN VIVO* RIBOSE SYNTHESIS IN THE RAT [1, 2]

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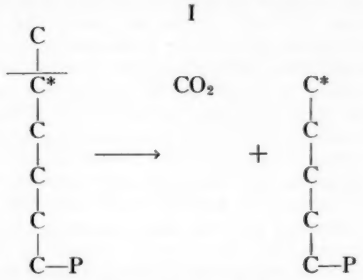
Ribose synthesis in animal tissue (1–3) has been demonstrated to take place via the oxidative and the nonoxidative reactions of the pentose phosphate pathway (4). The levels of the enzymes which catalyze the oxidative reactions of the pathway have been shown to be affected by hormonal alterations (5), and evidence has been presented indicating depression of the nonoxidative reactions in the tissues of thiamine-deficient animals (6). However, a systematic study of those factors influencing ribose production has not been possible because of the absence of a satisfactory method for obtaining this pentose from the living animal.

We have recently reported a convenient technique for inducing the urinary excretion of ribose by the rat, and have presented evidence that urinary ribose and that isolated from nucleic acid are synthesized via the same or similar pathways (7). This ribose "trapping" procedure is based on the observation of Tabor and Hayaishi (8) and of Karjala (9) that the administration of imidazoleacetic acid (ImAA) or of histamine to the rat is followed by the urinary excretion of ImAA riboside.

We have now used the ImAA riboside technique to study the effects of thiamine deficiency, partial hepatectomy, tumor growth, diabetes, hypopituitarism, and the administration of thyroxin, glucagon and adrenocorticotropic hormone (ACTH) on ribose synthesis. We have also obtained evidence for the direct incorporation of exogenous ribose into ImAA riboside. The results of these investigations are the subject of the present report.

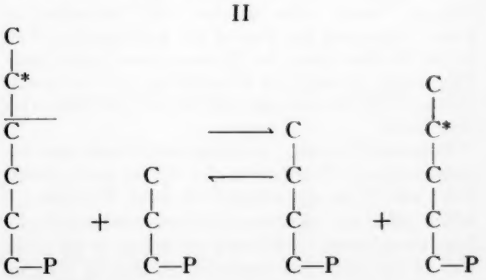
Most of the studies to be reported were carried out with glucose-2-C^{14} as the administered radioactive precursor of ribose. Hexose specifically labeled in its second carbon atom is useful in distinguishing ribose produced by the two reaction sequences of the pentose phosphate pathway. Ribose derived from the oxidative removal of carbon 1 of hexose-2-C^{14} phosphate (the C-1 oxidation pathway) is labeled in its first position:

I



On the other hand, ribose-2-C^{14} is produced in the nonoxidative reactions via the transketolase-catalyzed transfer of the first two carbons of hexose-2-C^{14} phosphate to an appropriate acceptor such as triose phosphate [3]:

II



[1] These studies were aided by grants from the Jane Coffin Childs Memorial Fund for Medical Research and the United States Public Health Service (C-3151).

[2] Presented in part at the forty-ninth annual meeting of the American Society for Clinical Investigation, Atlantic City, N. J., May 6, 1957.

[3] Ribose-2-C^{14} is also the product following the oxidative removal of carbon atom 6 of hexose-2-C^{14} (the C-6 oxidation pathway). However, recent studies have demonstrated that little if any pentose is synthesized from hexose via this mechanism (10).

In the normal rat ribose synthesized from glucose-2-C^{14} is predominantly labeled in carbon atoms 1 and 2, with the latter more radioactive than the former (2, 7). Carbon 4 is also labeled to an appreciable extent, presumably as a result of aldolase cleavage of hexose-2-C^{14} diphosphate and equilibration of the triose phosphates, followed by participation of triose-2-C^{14} phosphate in the transketolase reaction.

METHODS

ImAA riboside ribose isolation. The methods employed for the administration of imidazoleacetic acid and for the isolation and hydrolysis of ImAA riboside have been described in detail elsewhere (7). Briefly, each rat received three intraperitoneal injections at two hour intervals, except where otherwise noted. The injected fluid contained imidazoleacetic acid dihydrochloride, or histamine, sufficient sodium bicarbonate to effect neutrality, and a radioactive substrate, and was brought to a total volume of 15 ml. with isotonic saline. Rat 2 received ImAA and glucose-2-C^{14} by stomach tube. Urine was collected during the period of the injections and for the succeeding 16 hours. ImAA riboside was isolated by column chromatography, and hydrolyzed to free ribose and imidazoleacetic acid by incubation with an enzyme prepared from *Lactobacillus delbruckii* (11). The ribose was purified by column and paper chromatography, and then degraded by methods which have been summarized elsewhere (3), and which permit the isolation of each carbon atom of the pentose molecule as barium carbonate. The radioactivity of these samples was determined with a "micromil" end window counter with a counting efficiency of 13 per cent. Glucose was determined by the Nelson-Somogyi procedure (12, 13) and ribose by the orcinol method (14).

Animal preparation. Male rats of the Wistar or Sprague-Dawley strain with initial weights approximating 250 grams were used for these studies, except where otherwise noted. Most animals were maintained on Purina chow until the time of the first injection; Rats 13 and 14 were fasted for 72 hours prior to the study. Throughout the period of injections and for the ensuing 18 hours all of the rats were offered water *ad libitum* but were fasted.

Thiamine deficiency. In Experiment 3 the rat was maintained on a thiamine-free diet for six weeks, during which time it lost approximately 90 grams in weight. A fall in pulse rate, as measured electrocardiographically, from control levels of 550 beats per minute to 400 at the time of the study was considered evidence of profound thiamine deficiency (15). Rats 30 and 32 were young animals with initial weights approximating 100 grams, and received the thiamine-deficient diet and intraperitoneal injections containing 100 micrograms of oxythiamine daily for two weeks prior to the ImAA study. These animals gained 11 and 4 grams during the period of developing thiamine deficiency, as compared to weight gains of about 35 grams in litter mates receiving Purina chow.

Partial hepatectomy (Rats 4 and 5). Nine grams of liver was removed from Rat 4 under light ether anesthesia. ImAA administration and urine collection were begun 24 hours later. At the time of sacrifice 72 hours following partial hepatectomy, this animal's liver weighed 8.5 grams. The entire liver of a control unoperated rat of equal weight weighed 10.5 grams. Five grams of liver was removed from Rat 5, 72 hours prior to the study.

Diabetes mellitus (Rats 6, 7 and 8). Following a 48 hour fast these rats were injected subcutaneously with a freshly prepared, charcoal-treated solution containing 200 mg. of alloxan monohydrate per Kg. of body weight. Rat 6 was studied 48 hours, and Rats 7 and 8 seven days following alloxan administration. Rats 6 and 7 appeared very sick and had blood sugars of 650 and 800 mg. per 100 ml. The diabetes in Rat 8 was very mild (fasting blood sugar, at the conclusion of the study, was 150 mg. per 100 ml.).

Hormonal alterations (Rats 9, 10, 11 and 31). Rat 9 was made hyperthyroid by the daily subcutaneous injection of 0.5 mg. of sodium L-thyroxine pentahydrate for seven days prior to the study. During this period the animal lost 34 grams in body weight. Rat 10 was injected intraperitoneally with 4 units of adrenocorticotropic "gel" daily for eight days prior to the experiment. Rat 11 received 1 unit of crystalline glucagon (1 mg.) subcutaneously one-half hour before, and at hourly intervals during the period of imidazoleacetic acid administration, for a total of five injections. Rat 31 was hypophysectomized (Charles River Laboratories, Boston) one week prior to the study and received 5 per cent glucose in its drinking water. After the study the animal was sacrificed and autopsied; no tissue was found in the sella turcica.

Lactate and acetate injections. Rat 12 received 5, and Rat 13, 2 millimoles of sodium lactate, and Rat 14, 3.3 millimoles of sodium acetate in each of the three intraperitoneal injections containing the imidazoleacetic acid and glucose-2-C^{14}.

Tumor study. Rats 16 and 17 were each injected intraperitoneally with 1 ml. of the Yoshida hepatoma in the ascitic form. Rat 16 was studied 48 hours and 7 days later, and Rat 17, 10 days later. Massive ascites was present both in Rat 17 and in Rat 16 at the time of the second study. Both animals were given ImAA intraperitoneally, and Rat 17 received the glucose-2-C^{14} by the same route. To obviate complete glycolytic cleavage of the glucose-C^{14} by the tumor cells (16), Rat 16 was given the labeled sugar subcutaneously immediately following each intraperitoneal injection of ImAA.

Materials. Imidazoleacetic acid dihydrochloride was prepared according to the method of Bauer and Tabor (17). Glucose-2-C^{14} and ribose-1-C^{14} were obtained from the National Bureau of Standards through the courtesy of Dr. H. S. Isbell. Both the glucose (18) and the ribose

were degraded to their constituent carbon atoms and were found to contain radioactivity only in the carbon atom specified. Ribose, randomly labeled with C^{14} ("ribose-R-C^{14}"), was purchased from Schwarz Laboratories, and randomly-C^{14}-labeled glucose ("glucose-R-C^{14}") from New England Nuclear Corporation. The isotope patterns in the administered ribose and glucose are summarized in Tables III and IV, respectively. The specific molar activities of the radioactive sugars approximated 2 μc. per mg. A partial purification of *Lactobacillus delbruckii* riboside hydrolase was carried out according to the procedure of Takagi and Horecker (11). The thiamine-free diet and oxythiamine were purchased from Nutritional Biochemicals Company. A rat with the Yoshida hepatoma in ascites form was kindly provided by Dr. M. L. Stephenson of the Huntington Laboratories, Boston.

RESULTS

The data summarizing the fraction of administered imidazoleacetic acid or histamine excreted as ImAA riboside are presented in Table I. The administration of a total of 250 to 300 micromoles of ImAA to normal animals in three injections two hours apart was followed by the excretion of 22 to 31 per cent of ImAA as the riboside (Rats 1, 12 and 18). In a single experiment (Rat 2) in which ImAA was administered orally, 19 per cent was excreted as the riboside. Considerably less riboside was excreted by hepatectomized rats (Rats 4 and 5) and by severely diabetic animals (6 and 7). No untoward effects were observed following

TABLE I

Urinary riboside ribose excreted by rats given imidazoleacetic acid (ImAA) or histamine and C^{14}-labeled sugar

"Experiment"	Animal status	ImAA administered	Urinary ribose	*Per cent* administered ImAA	C^{14} administered		Molar activity of ribose	Total radioactivity in ribose	*Per cent* administered C^{14} in ribose
					Compound	Cpm $\times 10^6$			
		μM	*μM*				*cpm/μM*	*cpm*	
1	Normal	250	77	31	Glucose-2-C^{14}	2.86	202	15,600	0.54
2	Normal	250*	47	19	Glucose-2-C^{14}	1.43	166	7,800	0.55
3	Thiamine-deficient	250	60	24	Glucose-2-C^{14}	2.86	185	11,100	0.39
32	Thiamine-deficient (oxythiamine)	150	42	28	Glucose-2-C^{14}	0.95	188	7,850	0.82
4	24 hrs. after subtotal hepatectomy	150	15	10	Glucose-2-C^{14}	5.83	150	2,250	0.04
4	48 hrs. after subtotal hepatectomy	167	9.5	6	Glucose-2-C^{14}	2.94	100	950	0.03
5	72 hrs. after 50% hepatectomy	400	45	11	Glucose-2-C^{14}	5.83	167	7,500	0.13
6	Alloxan diabetes	250	8	3	Glucose-2-C^{14}	2.86	295	2,400	0.08
7	Alloxan diabetes	500	30	6	Glucose-2-C^{14}	8.59	635	19,000	0.22
8	Alloxan diabetes, mild	220	53	24	Glucose-2-C^{14}	5.29	368	19,500	0.37
9	Glucagon-treated	500	72	14	Glucose-2-C^{14}	7.15	666	48,000	0.67
10	ACTH-treated	250	53	21	Glucose-2-C^{14}	2.01	153	8,100	0.40
11	Hyperthyroid	300	40	13	Glucose-2-C^{14}	2.86	237	9,500	0.33
12	Normal, lactate-injected (15 mM)	250	55	22	Glucose-2-C^{14}	4.29	127	7,000	0.16
13	Normal, fasted 72 hrs., then injected with 6 mM lactate	500	69	14	Glucose-2-C^{14}	5.72	455	31,400	0.55
14	Normal, fasted 72 hrs., then injected with 10 mM acetate	500	101	20	Glucose-2-C^{14}	5.72	485	49,000	0.86
16	Yoshida ascites tumor, 48 hrs.	150	29	19	Glucose-2-C^{14}	4.17	158	4,500	0.11
16	Yoshida ascites tumor, 7 days	165	39	24	Glucose-2-C^{14}	5.72	282	11,000	0.19
17	Yoshida ascites tumor, 10 days	250	50	20	Glucose-2-C^{14}	2.86	150	7,500	0.26
18	Normal	300	84	28	Ribose-R-C^{14}	0.95	613	51,500	5.40
		Histamine administered (μM)		*Per cent* administered histamine					
19	Normal	313	66	20	Glucose-2-C^{14}	2.86	115	7,600	0.27
20	Normal	272	32	12	Ribose-1-C^{14}	1.14	1,420	45,500	4.00

* Imidazoleacetic acid given orally.

TABLE II

Isotope distribution in urinary ribose following administration of glucose-2-C^{14}

Experiment	Animal status	Relative radioactivity in ribose carbon atoms*				
		C-1	C-2	C-3	C-4	C-5
1	Normal	54	100	15	38	20
2	Normal	67	100	16	37	21
19	Normal (received histamine)	59	100	42	66	28
3	Thiamine-deficient	264	100		44	34
30	Thiamine-deficient (oxythiamine)	314	100	44	88	50
32	Thiamine-deficient (oxythiamine)	295	100			
4	24 hrs. after subtotal hepatectomy	68	100		45	18
4	48 hrs. after subtotal hepatectomy	90	100	16	48	13
5	72 hrs. after partial hepatectomy	97	100	16	40	23
7	Alloxan diabetes	64	100	17	38	14
8	Alloxan diabetes (mild)	49	100	16	20	9
9	Glucagon-treated	61	100			
10	ACTH-treated	82	100	19	40	20
11	Hyperthyroid	58	100		22	11
12	Lactate-treated	71	100	19	28	18
13	Lactate-treated	80	100			
14	Acetate-treated	63	100			
16	Yoshida ascites tumor, 2 days; C^{14}-glucose subcutaneously	84	100	20	38	12
16	Yoshida ascites tumor, 7 days; C^{14}-glucose subcutaneously	144	100		47	20
17	Yoshida ascites tumor, 10 days; C^{14}-glucose intraperitoneally	80	100	51	101	77
31	Hypophysectomized	65	100			

* Radioactivity in carbon 2 is given an arbitrary value of 100.

the administration of ImAA to any of these animals. Histamine administration (Rats 19 and 20) was also followed by substantial ImAA riboside excretion. The animals injected with histamine, however, were markedly somnolent for several hours following injection.

Table I also summarizes the data concerning isotope incorporation into ribose following the administration of C^{14}-labeled substrates. Ribose excreted by the normal rats given glucose-C^{14} contained 0.16 to 0.86 per cent of the administered radioactivity. In marked contrast, the two rats given ribose-C^{14} (18 and 20) incorporated 5.40 and 4.00 per cent of the injected C^{14} into the urinary pentose.

Table II depicts the isotope distribution in the urinary ImAA riboside ribose of the animals given glucose-2-C^{14}. There was a striking consistency in the radioactivity pattern in the pentose of all of the normal animals, with the C^{14} predominantly in carbon atoms 1 and 2, and the latter more active than the former. A variable fraction of the total radioactivity was distributed throughout the remainder of the ribose molecule with carbon 4 containing more isotope than positions 3 and 5. A striking departure from the normal carbon 1 to carbon 2 isotope ratio was seen in the thiamine-deficient animals (Rats 3, 30 and 32) where carbon 1 was as much as three times as radioactive as carbon 2. In Rats 4 (48 hour study) and 5, the rats with regenerating liver, carbon 1 approached carbon 2 in isotope content, and in one animal with massive ascites (16, seven day study), the activity in carbon 1 exceeded that in position 2. In Rat 17, the ascites tumor-bearing animal given glucose-C^{14} intraperitoneally, there was extensive isotope distribution throughout the ribose molecule.

TABLE III

Isotope distribution in urinary ribose isolated following administration of ribose-C^{14}

Experiment	Administered sugar	Relative radioactivity in isolated ribose carbon atoms*					Relative radioactivity in administered ribose carbon atoms*				
		C-1	C-2	C-3	C-4	C-5	C-1	C-2	C-3	C-4	C-5
20	Ribose-1-C^{14}	100	4	2	1	5	100	0	0	0	0
18	Ribose-R-C^{14}	80	100	109	68	46	100	100	122	78	54

* In Experiment 20 carbon 1, and in No. 18 carbon 2, are given arbitrary values of 100.

TABLE IV

Isotope distribution in urinary ribose isolated following administration of glucose randomly-labeled with C^{14}

Material	Relative radioactivity in sugar carbon atoms*					
	C-1	C-2	C-3	C-4	C-5	C-6
Isolated urinary ribose	84	121	100	87	68	
Administered glucose-C^{14}	111	97	114	100	89	80

* Carbon 3 of ribose and carbon 4 of glucose are given an arbitrary value of 100.

Table III describes the isotope distribution in the urinary ImAA riboside ribose of the rats injected with ribose-C^{14}. The urinary ribose from the rat (Rat 20) given ribose-1-C^{14} had 90 per cent of its isotope in carbon 1. The isotope patterns in ribose excreted by the rat (No. 18) administered randomly-labeled ribose (Table III) and by the animal given randomly-labeled glucose (Table IV) were not markedly different from those in the injected sugars.

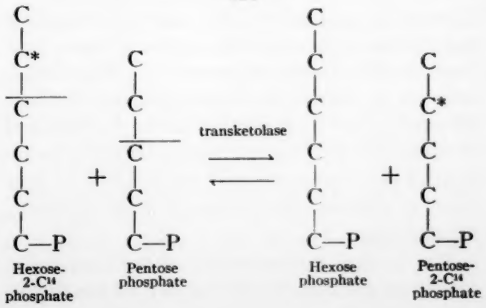
DISCUSSION

The advantages of the imidazoleacetic acid technique for studying ribose synthesis were described in a recent report (7). In addition to its relative ease, this method permits the separation of ribose with a much greater incorporation of administered radioactive carbon than is found in simultaneously isolated visceral nucleic acid ribose (7). The quantity of riboside excreted by our normal animals accounted for as much as 30 per cent of the administered ImAA. The one rat given ImAA orally excreted 19 per cent as the riboside. Tabor has shown that with sufficiently small doses of administered ImAA as much as 80 per cent is excreted as the riboside (19).

Although the site of riboside synthesis has not yet been determined, participation of the liver in this process is suggested by the substantial reduction in riboside excretion by the partially hepatectomized rats. The only other experimental condition which led to a very marked reduction in riboside excretion was severe diabetes. The presumed explanation for this observation is the low concentration in diabetic animals of glucose-6-phosphate, the precursor required for ribose synthesis. Whether the moderate reduction in ribose excretion by the hyperthyroid animal is significant will require further observations.

The pattern of isotope distribution in the ribose synthesized from glucose-2-C^{14} was remarkably consistent in all of the normal animals. The C^{14} in carbon atoms 1 and 2 of the ribose is considered to reflect ribose production from hexose carbon via the C-1 oxidation pathway, and the nonoxidative mechanism involving the transketolase reaction, respectively. The latter is known to require thiamine pyrophosphate as a coenzyme (20, 21), and an impairment of this reaction has recently been observed in the erythrocytes of thiamine-deficient rats (6). Our Experiments 3, 30 and 32 provide evidence for an interference with transketolase activity *in vivo* in the thiamine-deficient animal. The isotope ratio in ribose carbon atom 1 to that in carbon 2 of 2.6 to 3.1 in these rats, as compared with a ratio of 0.6 in normal animals, is consistent with a profound diminution in the transketolase reaction. Of great interest is the absence of a concomitant reduction in total ribose excretion by the thiamine-deficient rats. Two explanations for this phenomenon are available. One possibility is that sufficient additional ribose was synthesized by way of the C-1 oxidation pathway to compensate for the depression of the transketolase mechanism. A less likely reason stems from the suggestion that isotope normally found in carbon 2 of ribose derived from hexose-2-C^{14} may result from an exchange, rather than from a synthetic reaction:

III



If such were the case, an impairment of the transketolase reaction could result in an alteration of the isotope pattern in ribose without any change in net pentose production. However, the results of the studies of ImAA riboside synthesized from randomly-labeled ribose and from randomly-labeled glucose (see below) indicate that exchange reactions probably have little effect on isotope distribution in ribose synthesized from glucose-C^{14}.

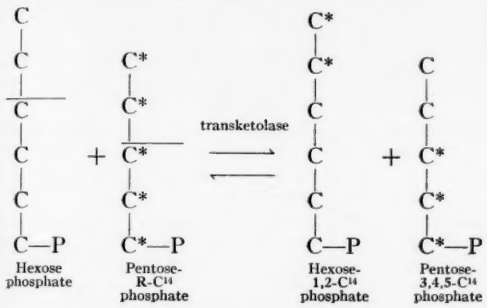
In the rats with regenerating liver the ribose carbon 1 to carbon 2 isotope ratio approached 1.0 and in one study in a tumor-bearing animal it exceeded 1.4. These observations may be interpreted in the light of our present concepts of the physiologic role of the glucose-6-phosphate and 6-phosphogluconic dehydrogenase reactions (4). These reactions, which result in the production of C-1 labeled pentose from C-2 labeled glucose, constitute the principal mechanism for the reduction of triphosphopyridine nucleotide (TPN). Since reduced TPN (TPNH) is required in a large number of reductive synthetic processes, including certain reactions involved in protein synthesis, an increased demand for TPNH might be anticipated in animals with regenerating liver or with rapidly growing tumors. Thus, the increased C-1 to C-2 ratio in these animals may reflect augmented requirements for TPNH. Further efforts to effect an increase in this ratio were made by attempting to stimulate other reactions in which TPNH is an essential cofactor. These include hexose synthesis from lactate (22), fatty acid synthesis from acetate (23), and steroid synthesis (24, 25). The administration of large quantities of lactate, acetate or ACTH, however, did not result in any shift in the ribose C-1 to C-2 ratio. Similarly, no change from the normal was observed in the isotope pattern of ribose synthesized by the thyrotoxic animal, although marked increases in the levels of liver glucose 6-phosphate and 6-phosphogluconic dehydrogenases have been found in thyroid-treated rats (5). The change from the normal in the isotope pattern of ribose obtained 48 and 72 hours, but not in that excreted 24 hours following partial hepatectomy may be related to the observation that regeneration of liver tissue is maximal from 48 to 96 hours following hepatectomy (26).

The marked randomization of radioactivity in the ribose excreted by the massive ascites tumor-bearing Rat 17 makes difficult conclusions from the C-1 to C-2 isotope ratio. The randomization may be ascribed to the fact that this animal received glucose-2-C^{14} intraperitoneally. It has previously been shown that small quantities of hexose injected intraperitoneally are almost completely cleaved to triose by ascites tumor cells (16). Hexose synthesized from triose-C^{14} which reaches the livers of ascites tumor-bearing animals shows extensive isotope randomization (16). Marked isotope distribution was also present in the ribose excreted by the normal rat given glucose-2-C^{14} and histamine (Experiment 19). It is likely that the profound depression observed in the histamine-treated animals was accompanied by alterations in many metabolic processes.

The animals given ImAA or histamine and ribose-C^{14} incorporated 4 to 5 per cent of the administered C^{14} in the urinary ribose as compared with an incorporation of less than 1 per cent by rats given glucose-C^{14}. These observations do not permit quantitative conclusions concerning the relative efficiency of the two sugars as riboside ribose precursors because of differences in the ribose and glucose pool sizes. However, the data do exclude the possibility that ribose-carbon must be converted to hexose prior to its incorporation into riboside. Further evidence against such a sequence of events is provided by the isotope distribution in the urinary ribose of Rat 20, the animal given ribose-1-C^{14}. The pattern of radioactivity in the urinary ribose in this experiment was very similar to that in the administered ribose. Had a prior conversion to hexose taken place, the isotope pattern in the urinary ribose would have been vastly different [ribose-1-C^{14} is converted to hexose-1,3-C^{14} both *in vitro* (27) and *in vivo* (28)]. Similar evidence was presented for direct incorporation of ribose-1-C^{14} into nucleic acid ribose of a human carcinoma cell grown in tissue culture (3).

Ribose randomly labeled with C^{14} was administered to Rat 18 in an effort to obtain information concerning the possible role of exchange reactions in determining the isotope pattern of urinary ribose. An interchange of 2 carbon fragments ("active glycolaldehyde") between nonradioactive hexose and randomly-C^{14}-labeled pentose phosphates would be expected to effect a dilution in the radioactivity in carbon 2 relative to carbon 3 of

the urinary ribose, as compared with the pattern in the starting material:



It is apparent that little change in the C-2 to C-3 ratio took place in our study. It is, of course, true that such an exchange reaction could occur only if ribose-5-phosphate were converted to xylulose-5-phosphate, the pentose which acts as the "active glycolaldehyde" donor in the transketolase reaction (*29*, 30), before all of the ribose-5-phosphate was consumed in riboside synthesis. However, the interconversion of the pentose phosphates is known to be rapid (31), and hence the phosphorylation of ribose would be immediately followed by the presence of an equilibrium mixture of the pentose phosphates. Further evidence against the possibility that exchange reactions have altered the isotope distribution is provided by the study of ribose synthesized from randomly-labeled glucose. Here again, the pattern of radioactivity in the urinary ribose is not markedly different from that in the starting material.

SUMMARY

Ribose synthesis *in vivo* has been studied by isolating imidazoleacetic acid riboside from rats given imidazoleacetic acid and a C^{14}-labeled sugar. Evidence is presented which indicates an impairment of riboside excretion in partially hepatectomized and in diabetic animals. The isotope distribution in ribose synthesized from glucose-2-C^{14} by normal animals is consistent with synthesis via both the oxidative and the nonoxidative reactions of the pentose phosphate pathway. Thiamine deficiency resulted in a marked decrease of ribose synthesis from hexose via the nonoxidative mechanism. An apparent increase in ribose production by way of the oxidative reactions was observed in rats with regenerating livers and in tumor-bearing animals. These observations are discussed in the light of our present concepts of the role of the oxidative reactions in TPNH production for synthetic processes. Evidence is presented for the direct incorporation of administered ribose into the urinary riboside.

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STUDIES OF RIBOSE METABOLISM. VI. PATHWAYS OF RIBOSE SYNTHESIS IN MAN [1]

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Although it has been demonstrated that the pathways involved in ribose biosynthesis from hexose by a human carcinoma cell grown in tissue culture (1) and by animals *in vivo* (2–4) are similar, little information is available concerning *in vivo* mechanisms of ribose production in man. The absence of a satisfactory technique for investigating this problem led us to explore the applicability to human studies of a recently described, convenient method for "trapping" ribose in the urine of the rat (4). This procedure, which was suggested by an observation of Tabor and Hayaishi (5), involves the administration of imidazoleacetic acid (ImAA) orally or parenterally, and the subsequent isolation of imidazoleacetic acid riboside from the urine. The present report is designed to summarize ImAA toxicity studies in several animal species and to demonstrate the usefulness of the ImAA procedure for investigations of ribose synthesis in man. This technique has already been employed to demonstrate a block in the metabolism of glucuronic acid by subjects with essential pentosuria (6).

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METHODS

Toxicity studies. Imidazoleacetic acid dihydrochloride was dissolved in water, neutralized with $NaHCO_3$, and administered by the routes indicated in amounts up to 3 millimoles per kilogram to mice, rats, guinea pigs and a dog. Most of the animals were watched closely for manifestations of toxicity for several hours, and then at intervals for several weeks following administration of ImAA. A guinea pig was sacrificed two weeks following injection, and specimens of several organs were excised for histologic study. ImAA was administered intravenously to a dog under light morphine anesthesia, and blood pressure, as measured with an in-lying femoral artery needle, and pulse and respiratory rates were determined for two hours thereafter.

Human studies. Three patients with short life expectancies were given ImAA and radioactive glucose or glucuronolactone. The first subject, Le, a 75 year old man with metastatic bronchogenic carcinoma, received by mouth 1,500 micromoles of ImAA dihydrochloride, an equimolar amount of sodium bicarbonate, and glucose-2-C^{14}, dissolved in 100 ml. of water, in three equal aliquots, 2 hours apart. The first dose was given 15 hours following the last ingestion of food (before breakfast), and the second and third doses, 2 and 4 hours following breakfast. Patient To, a 67 year old male with coronary artery disease, was treated in an exactly similar fashion. Patient Do, a 67 year old male with coronary artery disease, was given 2,000 micromoles each of imidazoleacetic acid and of sodium bicarbonate in a single dose by mouth,

TABLE I

Urinary ribose in human subjects given imidazoleacetic acid (ImAA)

Subject	Age	Diagnosis	ImAA administered	Urinary ribose	*Per cent* administered ImAA	C^{14} compound administered	Molar activity of ribose	C^{14} in ribose	*Per cent* administered C^{14} in ribose
	yrs.		*μM*	*μM*			*cpm/μM*	*cpm*	
Le	75	Bronchogenic carcinoma	1,500	460	31	Glucose-2-C^{14} (2.86×10^7 cpm; 50 mg.)	32.3	1.42×10^4	0.050
To	67	Coronary artery disease	1,500	570	38	Glucose-2-C^{14} (2.86×10^7 cpm; 50 mg.)	26.0	1.48×10^4	0.052
Do	67	Coronary artery disease	2,000	275	14	Glucuronolactone-U-C^{14} (1.57×10^7 cpm; 67 mg.)	33.0	9.1×10^3	0.058

TABLE II

Isotope distribution in urinary ribose of subjects given imidazoleacetic acid and glucose-2-C¹⁴

Subject	Per cent total radioactivity in ribose carbon atoms				
	C-1	C-2	C-3	C-4	C-5
Le	51	33	3	9	4
To	50	39	3	6	2

and 15 minutes later, an intravenous injection of 5 ml. of isotonic saline containing D-glucuronolactone-U-C¹⁴. None of these subjects had evidence of azotemia, proteinuria or liver disease, and each had a normal fasting blood glucose.

Urine was collected in two aliquots for 24 hours following administration of the imidazoleacetic acid. All of the riboside was found in the sample collected during the first 10 hours. The procedure for isolating rat urinary ribose from the urinary imidazoleacetic acid riboside was described in detail elsewhere (4). The method used here was similar, except that instead of using the entire urine collection, one-third of the urine excreted during the first 10 hours was acidified, aerated to remove CO_2, brought to pH 8, and adsorbed on Dowex-1, 100 to 200 mesh, 2 per cent cross-linked, acetate form (height, 16.0 cm.; diameter, 4.5 cm.). The column was washed with water until the effluents were free of radioactivity, and the riboside was then eluted with 0.2 N acetic acid. The eluates were collected in 50 ml. fractions, and the riboside was found in fractions 5 and 6. These were combined and treated as described previously (4). The ribose was degraded to its constituent carbon atoms by methods that have been summarized elsewhere (1). Radioactivity was measured with a "micromil" end window counter with an efficiency of 13 per cent.

Materials. Imidazoleacetic acid dihydrochloride was synthesized according to the procedure of Bauer and Tabor (7). All of the toxicity studies were carried out on the batch of ImAA subsequently used for the human studies. Uniformly C¹⁴-labeled glucuronolactone (glucuronolactone-U-C¹⁴), which was kindly provided by Dr. N. E. Artz of the Corn Products Refining Company, Argo, Ill., was dissolved in 0.9 per cent saline and sterilized by passage through a bacteriologic filter. This material was found to give a single radioactive spot on descending chromatography in butanol : ethanol : water (52 : 32 : 16).

Glucose-2-C¹⁴ was purchased from the National Bureau of Standards through the courtesy of Dr. H. S. Isbell. Degradation of the glucose to its constituent carbon

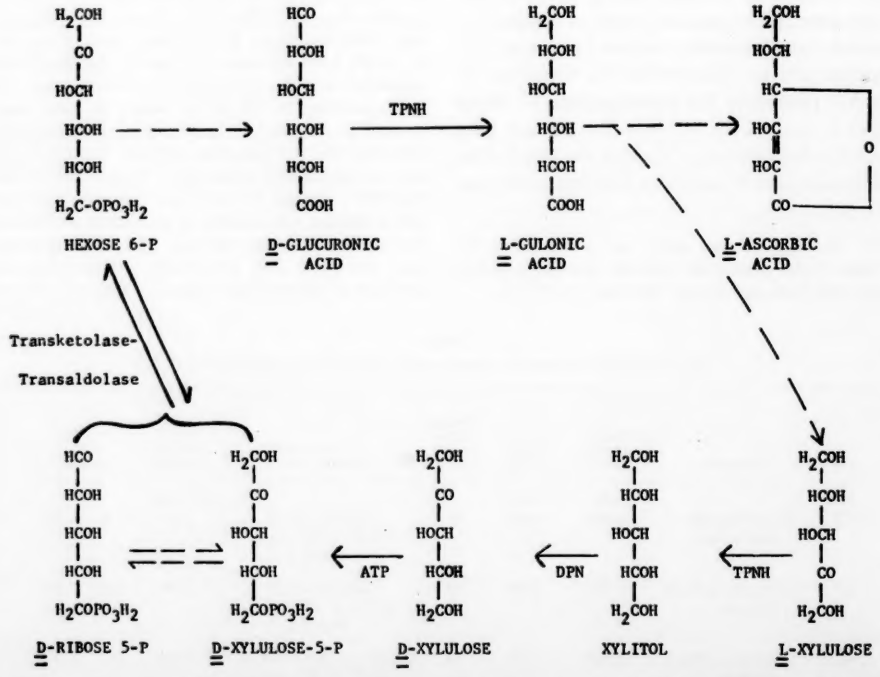

FIG. 1. GLUCURONIC ACID CONVERSION TO HEXOSE PHOSPHATE VIA THE C-6 OXIDATION AND PENTOSE PHOSPHATE PATHWAYS

atoms by methods previously described (8) revealed that its radioactivity was limited to carbon atom 2.

RESULTS

Toxicity studies

The intraperitoneal administration of 2 millimoles of ImAA per Kg. of body weight to mice and to rats had no discernible toxic effect. The administration of 3 and 4 millimoles per Kg. to mice led to marked depression for 12 hours followed by apparently complete recovery. The intraperitoneal injection of as much as 8.5 millimoles per Kg. to guinea pigs produced no detectable untoward effect. A guinea pig given 4 millimoles per Kg. intraperitoneally and sacrificed two weeks later had no demonstrable abnormalities in histologic sections of lung, liver, kidney, heart and adrenal glands. The intravenous administration of 11.2 millimoles in 20 ml. of distilled water in 1 minute to a 17 Kg. dog under light morphine anesthesia led to a rise in pulse rate of 10 beats per minute, with no effect on blood pressure or respiration. The administration of an equal amount of ImAA to the same animal 30 minutes later led to no effect on any of these measurements. No immediate or delayed toxic effects were observed in the human subjects given imidazoleacetic acid.

Ribose synthesis

The human subjects excreted up to 570 micromoles of ribose as ImAA riboside (Table I). The amount of riboside excreted was greatest in Subjects To and Le, to whom the ImAA was given in divided doses over a period of several hours. In all three subjects there was appreciable C^{14} incorporation into the urinary ribose from glucose-2-C^{14} or glucuronolactone-C^{14} (Table I).

The isotope distribution in the urinary ribose of the two subjects given glucose-2-C^{14} is summarized in Table II. The preponderance of radioactivity was found in carbon atoms 1 and 2, with the former more active than the latter.

DISCUSSION

These studies indicate that as much as 2 to 8.5 *millimoles* of imidazoleacetic acid per Kg. of body weight can be given to some animal species without discernible toxicity. The oral administration to human subjects of less than 30 *micromoles* per Kg., on the other hand, permitted the isolation of sufficient ribose for studies of intermediary metabolism, and resulted in no untoward effects. When the ImAA was administered in divided doses over several hours, as much as 38 per cent was excreted as the riboside. However, in the patient given ImAA in a single dose only 19 per cent appeared in the urine in the glycosidic form. Tabor has previously shown that in rats given sufficiently small doses of imidazoleacetic acid as much as 80 per cent may be excreted as the riboside (9).

Isotope distribution in ribose synthesized from glucose-2-C^{14} is consistent with synthesis via the oxidative and nonoxidative mechanisms of the pentose phosphate pathway (10). The preponderance of isotope in carbon 1 as compared with carbon 2 is the reverse of the situation observed in a human carcinoma cell in tissue culture (1), and in normal growing rats (3, 4). This suggests that in our human subjects ribose production occurred predominantly by way of the C-1 oxidation pathway (the glucose 6-phosphate and 6-phosphogluconic dehydrogenase reactions). The relatively small amount of isotope randomization to carbon atoms 3, 4 and 5 indicates that little dissimilation of the hexose molecule took place prior to ribose synthesis. The slight isotope excess in carbon 4 as compared with positions 3 and 5 is consistent with the aldolase cleavage of hexose-2-C^{14} phosphate to triose phosphates, and equilibration of the trioses, prior to triose phosphate participation in the transketolase reaction.

The implications of the study demonstrating ribose synthesis from glucuronolactone-C^{14} have been discussed in detail elsewhere (6, 11). Briefly, this experiment is considered to demonstrate the operation in man of the recently described C-6 oxidation pathway (10) (Figure 1). This pathway is utilized by most mammalian organisms for the synthesis of ascorbic acid (12–15). Man, however, is unable to synthesize the vitamin and presumably employs this pathway only to permit the return of glucuronic acid carbon to the mainstream of carbohydrate metabolism, with the pentose, L-xylulose, as an intermediate (16). A block in this pathway has been postulated (16) to account for the urinary excretion of L-xylulose by subjects with the genetic disorder, chronic essential pentosuria (17). Strong support for this hypothesis

was obtained in a recent study of ribose excretion by a pentosuric subject given imidazoleacetic acid and glucuronolactone-C^{14} (6). In striking contrast to Subject Do, who excreted ribose containing 0.058 per cent of the radioactive carbon present in the administered glucuronolactone, a pentosuric subject excreted ribose which was virtually free of radioactivity. The pentosuric individual, however, excreted 4,000 micromoles of L-xylulose, which contained over 4 per cent of the administered C^{14}. The radioactivity in the L-xylulose demonstrated its origin from D-glucuronolactone, while the absence of C^{14} in the urinary ribose indicated that it was derived from sources other than the administered lactone. These data provide evidence for an impairment in the metabolism of glucuronic acid beyond the L-xylulose step in essential pentosuria.

SUMMARY

A technique is described for the study of ribose synthesis in man. This involves the oral administration of imidazoleacetic acid, which has been shown to be of exceedingly low toxicity, and the isolation of ribose from urinary imidazoleacetic acid riboside. Isotope distribution in ribose synthesized from glucose-2-C^{14} by individuals with normal carbohydrate metabolism is consonant with ribose biosynthesis from hexose via the oxidative and the nonoxidative reactions of the pentose phosphate pathway. An additional mechanism of ribose biosynthesis, from glucuronic acid, is indicated by the excretion of ribose-C^{14} by a human subject given imidazoleacetic acid and D-glucuronolactone. A block in this pathway has previously been shown to be responsible for the excretion of L-xylulose by individuals with chronic essential pentosuria.

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